

STRUCTURE-FUNCTION STUDIES OF MURINE GAMMA INTERFERON: A  
SYNTHETIC PEPTIDE AND ANTIBODY APPROACH

BY

MICHAEL ANDREW JARPE

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## TABLE OF CONTENTS

|   | <u>page</u> |
|---|-------------|
| ACKNOWLEDGEMENTS.....   | ii          |
| ABSTRACT.....   | iv          |
| CHAPTERS  |             |
| 1. INTRODUCTION   |             |
| Interferons: Historical Perspective.....  | 1           |
| IFN- $\gamma$ Function.....   | 5           |
| Structure/Function Studies of IFN- $\gamma$ .....   | 8           |
| The Synthetic Peptide Approach.....   | 11          |
| Summary and Objective.....  | 14          |
| 2. STRUCTURE OF AN EPITOPE IN A REGION OF THE IFN- $\gamma$<br>MOLECULE THAT IS INVOLVED IN RECEPTOR<br>INTERACTION |             |
| Introduction.....   | 16          |
| Material and Methods.....   | 17          |
| Results and Discussion.....   | 24          |
| 3. TOPOLOGY OF RECEPTOR BINDING DOMAINS OF MOUSE IFN- $\gamma$<br>THAT INCLUDE THE N-AND C-TERMINI.                 |             |
| Introduction.....   | 46          |
| Materials and Methods.....  | 47          |
| Results and Discussion.....   | 52          |
| 4. SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS .....  | 71          |
| LIST OF REFERENCES .....  | 76          |
| BIOGRAPHICAL SKETCH .....   | 89          |

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Michael Andrew Jarpe

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Chairman: Howard M. Johnson

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Gamma Interferon (IFN- $\gamma$ ) is an important immunoregulatory lymphokine that affects a wide variety of immune functions. I have undertaken studies to elucidate the structural basis for IFN- $\gamma$  function. These structure/function studies of IFN- $\gamma$  have yielded data on the location of functional domains.

A monoclonal antibody (mAb) specific for the N-terminus of mouse IFN- $\gamma$  was shown to neutralize IFN- $\gamma$  function by blocking receptor binding. I mapped the epitope specificity of this monoclonal antibody to residues 3 through 14. Additionally, residues at positions 3, 4, and 5, are particularly important in the structure of the epitope, since peptides that lacked these residues had reduced ability to interact with the mAb. Furthermore, peptide analogs that

replaced these residues with nonconservative substitutions lost antibody binding activity. Tyrosine in position 14 was also found to be critical, since peptides lacking it also lost binding activity. The epitope specificity for this mAb encompasses the 12 residues between 3 and 14 and is linear and discontinuous. Because this mAb blocks IFN- $\gamma$  receptor binding, this epitope may be involved in receptor interaction.

Antibodies raised against five overlapping synthetic peptides that encompass the entire sequence of mouse IFN- $\gamma$  were tested for their ability to inhibit IFN- $\gamma$  function. Only antisera raised against the N-terminal and the C-terminal peptides could neutralize IFN- $\gamma$  by blocking receptor binding. Further mapping studies showed that the C-terminal specific antiserum was directed to several neutralizing epitopes within the region. The antibody neutralization data suggest that IFN- $\gamma$  contains two binding domains, one in the N-terminus and one in the C-terminus. A three dimensional model of IFN- $\gamma$  has been constructed utilizing both functional data, predictive algorithms for secondary and tertiary structure, and comparison to the known structure of IL-2. The molecule is predicted to form six  $\alpha$ -helices divided by five turns and to form a four-helix bundle motif. The N-terminus and C-terminus form two receptor binding domains and are found close together. This model provides a working base for future studies.

## CHAPTER 1 INTRODUCTION

### Interferons: Historical Perspective

Interferon (IFN) was first discovered in 1957 by Isaacs and Lindenmann (1) as a substance that had the ability to protect host cells against viral infection. The term interferon has subsequently come to describe a class of proteins that fall into three distinct groups designated as IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\gamma$ . IFN- $\alpha$  and IFN- $\beta$  are classically produced in cells induced by viruses or polyribonucleotides (2). IFN- $\gamma$  is classically produced by T-lymphocytes and natural killer (NK) cells in response to antigen or mitogen stimulation (2).

Genes for all three classes of IFNs have been cloned and sequenced. IFN- $\alpha$  and IFN- $\beta$  have about 29% sequence homology while IFN- $\gamma$  has little homology to IFN- $\alpha$  or IFN- $\beta$ . IFN- $\alpha$  and IFN- $\beta$  have been shown to exist in most vertebrates; however, IFN- $\gamma$  has been shown to exist only in mammals (3). IFN- $\alpha$  is actually a family of 23 genes each without introns and 15 of these genes encode for full length proteins of between 165 and 172 amino acid residues (4). There are three IFN- $\beta$  genes, one without introns coding for a protein of 166 amino acid residues and two with four introns each (5-10). The

genes containing introns are called IFN- $\beta$ 2 or IL-6 (5). IFN- $\beta$ 2 has been shown to be a B-cell differentiation factor (originally called BSF-2) that can induce B-cell proliferation and enhance antibody production (6,8). Although IFN- $\beta$ 2 activity can be neutralized by antiserum to IFN- $\beta$ , IFN- $\beta$ 2 bears little homology to the other IFN- $\beta$  molecules and shows little antiviral activity, therefore its classification as an IFN is debatable (11).

IFN- $\gamma$  has been cloned in human (12,13), mouse (14), rat (15), and bovine (16) and is encoded by a gene with three introns and it appears that there is only one IFN- $\gamma$  gene present in the genome. A strong species specificity exists with IFN- $\gamma$  (11). Table 1-1 illustrates the amino acid sequence homology between the sequences of the four known IFN- $\gamma$  proteins from human, cattle, rat and mouse. The sequence analysis was performed on a microVAX using sequence data from the GenBank database and the program GAP. GAP aligns sequences and produces a best fit by inserting gaps within the sequences. The program reports the percentage of residues that are identical between sequences and also reports the percentage similarity. Similarity is defined as the percentage of identical residues plus the percentage of residues that are conservative substitutions. In most of the comparisons the percentage identity is low. For example, when human IFN- $\gamma$  and mouse IFN- $\gamma$  are compared, there is a 40% identity (Table I). However, the percentage similarity is 67%. Sixty-seven percent similarity indicates there are many

conservative amino acid substitutions. This same trend appears in all of the comparisons. The lack of homology may account for the species specificity. Based on the degree of similarity, it is likely that the proteins are structurally similar. This suggests that information concerning the location of functional domains in one species may be applicable to other species.

A receptor for IFN- $\gamma$  has been identified, cloned and sequenced in both human and mouse (17-21) and shown to exist on chromosome six in the human (22). The deduced amino acid sequence yields a protein of 451 and 472 amino acids for mouse and human, respectively (17-21). There is about 55% overall homology between the mouse and human proteins (18-21). A predicted transmembrane domain divides the protein in half and both domains bear no appreciable homology to any known receptors (17-21). The external domain comprises the binding domain and when expressed on the surface of a cell exhibits identical binding characteristics as the native receptor which suggest that there is only one binding protein for IFN- $\gamma$  (17-21). The expressed protein has no function when IFN- $\gamma$  binds to it (17-21). However, if the short arm of human chromosome 22 is present in hamster/human hybrid cells in addition to the receptor, function is restored, suggesting that there is some other factor required for functional activity in addition to binding of IFN- $\gamma$  (23). The requirements of the receptor for binding and the requirements

Table 1-1. Amino acid sequence homology between known IFN- $\gamma$  proteins from different species.

| Species | Human  | Bovine | Rat    |
|---------|--------|--------|--------|
| Bovine  | 62(76) | -      | -      |
| Rat     | 37(60) | 45(68) | -      |
| Mouse   | 40(67) | 43(67) | 86(95) |

Values are percent amino acid homology. Values in parentheses are percent homology plus percent conservative substitutions.

of other as yet undiscovered factors for receptor function need further study.

#### IFN- $\gamma$ Function

IFN- $\gamma$  is a glycoprotein of approximately 20,000 daltons that possesses many diverse activities. IFN- $\gamma$  was classically described as a product of white blood cells stimulated with phytohaemagglutinin, a T-cell mitogen, that possessed antiviral activity (24). Later, this product was shown to be different from the type I interferons (IFN- $\alpha$  and IFN- $\beta$ ) in its instability at pH 2 (24). IFN- $\gamma$  was also found to be antigenically different from type I IFNs (25). These characteristics, in part, classify it as type II IFN or immune IFN. The observation that IFN had immunomodulatory activities was initially made with IFN- $\alpha$  and IFN- $\beta$  when it was shown that they had a suppressive effect on antibody production (26-31). IFN was also shown to regulate delayed type hypersensitivity (32-36), cellular immunity (32-36), and natural killer cytotoxic activity (37-40). IFN- $\gamma$  was shown to possess immunomodulatory activities, some in common to and others unique from IFN- $\alpha$  and IFN- $\beta$  (41). In fact, the immunoregulatory potency of IFN- $\gamma$  is much greater than that of IFN- $\alpha$  and IFN- $\beta$  (42).

IFN- $\gamma$  has the ability to modulate antibody production by B lymphocytes both positively and negatively (43-49). IFN- $\gamma$  was shown to suppress in vitro antibody response, in mice,

before clonal expansion of B-cells had occurred (44,45,47). Conversely, IFN- $\gamma$  had a positive effect when added to B-lymphocytes that had already undergone clonal expansion by inducing antibody secretion and terminal differentiation (43,46,48,49).

The increased expression of a variety of cell surface molecules can be achieved with IFN- $\gamma$ . IFN- $\gamma$  can increase the expression of interleukin-2 receptors on lymphocytes (50). IFN- $\gamma$  can increase surface expression of Fc receptors on macrophages which are involved in antibody binding and antibody dependent cell cytotoxicity (51,52). Class-I major histocompatibility antigen expression is increased by IFN- $\gamma$  (53,54). Class-I expression is required for T-cell recognition for the killing of virally infected cells. Class-II molecule expression, which is critical for antigen presentation and the induction of an immune response, is also increased by IFN- $\gamma$  (55,56).

Another immunomodulatory function of IFN- $\gamma$ , and possibly one that has received the most attention, is the activation of a variety of cytotoxic cells that have the ability to kill tumor cells. Both NK cell activity and cytotoxic T-cell activity are increased by IFN- $\gamma$  (37-40,57). The tumoricidal activity of macrophages has been shown to be augmented by factor(s) liberated from antigen or mitogen stimulated lymphocytes and has been termed macrophage activating factor or MAF (58-62). The process of activation actually occurs in a series of steps, beginning with priming of macrophages by

MAF then subsequent triggering by a small amount of lipopolysaccharide (LPS) (60, 61). Macrophages so treated have a greatly enhanced ability to kill tumor cells in vitro. MAF activity has been shown to be mainly, if not exclusively, due to IFN- $\gamma$  (59, 60, 63). Recombinant or purified natural IFN- $\gamma$  has potent MAF activity, and IFN- $\gamma$  neutralizing antibodies can remove MAF activity completely from MAF containing cultures (60, 63). Both IFN- $\alpha$  and IFN- $\beta$  have MAF activity (58), although they are much less potent when compared with IFN- $\gamma$  (64).

The in vivo properties of IFN- $\gamma$  have been studied primarily in the context of its potential anti-cancer properties. Studies have shown, at least in experimental animal systems, that IFN- $\gamma$  has a direct role in host tumor defense (65, 66). In a mouse tumor model where tumors can be implanted that either progress or spontaneously regress when the dose of tumor cells is varied, higher levels of IFN- $\gamma$  were observed in regressing tumors (65). Additionally, when an IFN- $\gamma$  neutralizing monoclonal antibody (mAb) was injected intraperitoneally into mice that possessed regressing tumors, the tumors were converted to progressing tumors. A non-neutralizing mAb that bound IFN- $\gamma$  but did not neutralize its function had no effect on tumor growth. Others have shown similar results using direct tumor injection of IFN- $\gamma$  neutralizing mAbs in similar tumor models (66). These finding suggest that IFN- $\gamma$  may be functioning in host tumor defense under normal circumstances, but when administered in

clinical trials, IFN- $\gamma$  seems to be ineffective in treating tumors (67-74). The reason for its ineffectivity is unknown.

#### Structure/Function Studies of IFN- $\gamma$

When the genes for human and mouse IFN- $\gamma$  were cloned in the early 1980s and the primary sequence was determined, it was thought that an understanding of the mechanism for IFN- $\gamma$  function would soon follow. To the contrary, the structure/function relationship of IFN- $\gamma$  has proven to be more complicated than first thought. Studies designed to explore this area have involved the use of a variety of techniques and can be divided into approaches using molecular biology, antibodies, synthetic peptides, and combinations thereof. Some of these studies have yielded conflicting results.

Some studies have shown that IFN- $\gamma$  may exist as homodimer (75-77) or larger multimer (78); however, the exact role of these multimer structures is unclear. It has been shown that IFN- $\gamma$  isolated in its monomer form has activity (75). Recombinant IFN- $\gamma$  expressed in *E. coli* lacks glycosylation but retains full activity (12,14) which shows that glycosylation is not required for IFN- $\gamma$  activity. It has been postulated that glycosylation of IFN- $\gamma$  provides protection of the mature protein against proteolysis (75). Initially, the amino (N) terminus of IFN- $\gamma$  was thought to be the amino acid sequence CYS-TYR-CYS which left the

possibility of disulfide bond formation (12,14). This possibility was rejected when N-terminal sequencing of human natural IFN- $\gamma$  showed that the N-terminus was pyroglutamic acid (75). Therefore, no disulfide bonding occurs in IFN- $\gamma$ .

Structure/function studies have been carried out with IFN- $\gamma$  using truncated molecules generated through mutation of cDNA clones or through limited proteolysis of recombinant IFN- $\gamma$  (79-83). Human and mouse IFN- $\gamma$  lacking the N-terminal eight or nine amino acid residues have been shown to have significantly reduced functional activity and reduced receptor binding activity (79-80). This abrogation of activity in human IFN- $\gamma$  was shown to correlate to a significant loss in  $\alpha$ -helical structure (80). Recombinant human IFN- $\gamma$  lacking portions of its C-terminus had either a significant reduction in activity (81,83) or no change in activity (82). Human recombinant IFN- $\gamma$  treated with proteases that removed 11 (81) or 13 (83) residues from the C-terminus showed a significant decrease in IFN- $\gamma$  function and receptor binding activity. Conversely, a mutant recombinant IFN- $\gamma$  from *E. coli* that lacked the 23 C-terminal residues retained full activity (82). The reason for this discrepancy remains unclear and may have something to do with proteolytic attack altering more than just the C-terminus, although in each proteolytic experiment N-terminal sequencing confirmed that the N-terminus was not altered (81,83).

The use of antibodies, both polyclonal and monoclonal, to probe for functional sites along the IFN- $\gamma$  molecule has

proven useful in the study of the structure/function relationship of IFN- $\gamma$ . Most of these studies hint at a two or multiple functional domain model of IFN- $\gamma$ ; however, controversy surrounds the issue of where these domains lie. Some evidence exists that suggests that there are different functional domains for different IFN- $\gamma$  functions such as antiviral activity and macrophage priming activity (84); however, this has yet to be confirmed. One study using 21 neutralizing mAb to human IFN- $\gamma$  supports a multi-domain model (85). These mAb were divided into two groups using antibody-antibody competition assays with each group having a different epitope specificity, suggesting more than one functional domain. The location of these epitopes is unknown as they have not been mapped. Other studies have either localized mAb epitopes or raised site directed antibodies to synthetic peptides of the IFN- $\gamma$  molecule. Antibodies raised against a synthetic peptide corresponding to the first 20 residues of human IFN- $\gamma$  (the first three residues were CYS-TYR-CYS) neutralized IFN- $\gamma$  activity, suggesting that the N-terminal region of IFN- $\gamma$  was involved in function (86). A polyclonal antiserum raised against a synthetic peptide corresponding to the first 39 residues of mouse IFN- $\gamma$ , IFN- $\gamma$ (1-39), neutralized IFN- $\gamma$  activity by blocking IFN- $\gamma$  receptor binding (87). Additionally, the epitope specificity of a mAb shown to neutralize mouse IFN- $\gamma$  by blocking receptor binding was mapped preliminarily to residues 1-20 of the N-terminus of the molecule (88), supporting the role of the N-

terminus as a functional domain. Others have demonstrated that two mAb specific for the N-terminus of mouse IFN- $\gamma$  also can neutralize IFN- $\gamma$  activity (84). In a more direct experiment the synthetic peptide IFN- $\gamma$ (1-39) was able to block directly IFN- $\gamma$  function and compete specifically with IFN- $\gamma$  for its receptor (88). Thus, the role of the N-terminus in receptor binding seems clear.

Polyclonal antiserum raised against the C-terminal peptide of mouse IFN- $\gamma$ , IFN- $\gamma$ (95-133), blocks IFN- $\gamma$  function and receptor binding (87). The peptide, IFN- $\gamma$ (95-133), did not compete with IFN- $\gamma$  directly (88). Two C-terminal specific mAb can also neutralize mouse IFN- $\gamma$  activity (84). In contrast, studies have shown that mAb specific for the C-terminus of human IFN- $\gamma$  are unable to neutralize IFN- $\gamma$  activity and have concluded that the C-terminus is not important (89). However, the epitope specificities of these mAb have been directed to the last 15 residues of IFN- $\gamma$  and may have missed an important functional site.

#### The Synthetic Peptide Approach

The synthetic peptide approach has generated much information with regard to the location of functional domains on a number of different proteins. This approach can be divided into three related methods that utilize synthetic peptides to probe for functional domains. The first method involves the use of synthetic peptides to directly interact

with receptors or other molecules. Synthetic peptides corresponding to portions of ligands have been shown to compete directly with the larger intact molecule, thereby identifying a region critical for receptor interaction. A synthetic peptide corresponding to residues 400-411 of gamma chain human fibrinogen was shown to bind to fibrinogen receptor on platelets and block fibrinogen binding to its receptor (90). A site of interaction on the superantigen staphylococcal enterotoxin A (SEA) to class II molecules was found to be contained within the first 45 residues of SEA using direct peptide competition (91). These examples illustrate that synthetic peptides can act as direct probes for functional domains on proteins.

A second facet of the synthetic peptide approach is the use of synthetic peptides to map the epitope specificity of mAb that have been raised to an intact protein. Monoclonal antibodies that have been shown to interfere with the activity of a molecule are presumably binding to a region on the molecule that is important for its activity. Synthetic peptides can then be used to determine the epitope specificity of the mAb and thereby identify an important functional region. An example of this method is the determination of a functional site on the interleukin-2 (IL-2) molecule (92). Monoclonal antibodies raised against IL-2 were shown to inhibit its function and were mapped to the between residues 8-54 of the molecule using synthetic peptides.

A third part of the synthetic peptide approach involves the use of synthetic peptides to produce site-specific antibodies to regions of proteins. These antibodies, both polyclonal and monoclonal, can interact with the native protein and block its activity by interfering with an important domain. This technique has been successful in a number of different systems. In the study of IFN- $\gamma$ , antisera raised to a synthetic peptide corresponding to the N-terminal 20 residues of human IFN- $\gamma$  neutralized IFN- $\gamma$  activity (86). Polyclonal antibodies produced to a peptide from the 15 N-terminal residues of human tumor necrosis factor neutralized its activity (93). Antibodies raised to synthetic peptides of erythropoietin located a functional domain in the region between residues 99 and 129 (94).

As described, all of these uses of the synthetic peptide approach have been successful at locating functional sites on proteins. The interpretation of the results obtained from these studies is important. Studies where synthetic peptides can directly interact with receptors are easily interpreted. The peptide contains all or part of a functional domain of the protein. The identification of a synthetic peptide with these properties is not always possible. Some peptides do not adopt sufficient conformation in solution to be able to react with receptors. Our laboratory has found that longer peptides have a greater likelihood of possessing stable secondary structure and a greater chance of direct interaction. When direct peptide competition is not found,

the use of antibodies as probes for functional domains can be successful. The antibody molecule, by binding to a region critical for activity, can interfere with the function of the protein studied. One important drawback to this approach is the possibility that the antibody may be inducing a conformational change in the molecule that disrupts a domain in a distant region. Another is the possibility that the antibody may only bind to a subset of denatured molecules. One way to minimize these potential problems is to use a combination of the approaches discussed. This would increase the likelihood of identifying a true functional domain.

#### Summary and Objective

At present, the state of knowledge concerning IFN- $\gamma$  is one in which a large amount of information has been generated about it but there remain questions concerning the number, location, and characterization of functional domains. From earlier work with molecular biology, antibodies, and synthetic peptides there appears to be at least two distinct functional domains of IFN- $\gamma$ . These domains are believed to be located in the N-terminus and the C-terminus of the molecule. However, little is known about the functional role of the internal regions of the sequence. Studies with site directed mutation of human IFN- $\gamma$  provide some suggestive evidence that the internal region is critical to IFN- $\gamma$  function, at least by being involved in the maintenance of

tertiary structure (95). Single amino acid substitutions in the core of the molecule reduced receptor binding and structure. Also, little has been done to further characterize these potential domains with regard to precise mapping of important regions.

It is the objective of this dissertation to further the understanding of the structure/function properties of IFN- $\gamma$  by attempting to identify other functional domains and by further characterization of those that are known. This will be accomplished through two main specific aims. First, the N-terminal functional domain will be further characterized by mapping the precise epitope specificity of an N-terminal specific mAb that neutralizes IFN- $\gamma$  by blocking receptor binding. Second, other regions of the molecule will be explored for functional significance using long overlapping synthetic peptides encompassing the entire sequence of IFN- $\gamma$  and antiserum raised to these peptides.

CHAPTER 2  
STRUCTURE OF AN EPITOPE IN A REGION OF THE IFN- $\gamma$  MOLECULE  
THAT IS INVOLVED IN RECEPTOR INTERACTION

Introduction

The N-terminal region of IFN- $\gamma$  has been shown to contain a binding site for the IFN- $\gamma$  receptor based on specific blockage of IFN- $\gamma$  receptor binding by a synthetic peptide corresponding to the first 39 residues of IFN- $\gamma$  and based on blockage of IFN- $\gamma$  function by the N-terminal 39 residues as well as the first 20 residues of IFN- $\gamma$  (88). A monoclonal antibody has been produced that has been shown to neutralize IFN- $\gamma$  function (88). This mAb neutralizes IFN- $\gamma$  activity by blocking IFN- $\gamma$  binding to its receptor (88). This suggests that this mAb binds to a functional site on the IFN- $\gamma$  molecule that is involved in receptor interaction. The epitope to which this mAb binds is partially mapped to the first 20 residues of IFN- $\gamma$  using synthetic peptides as competitors for binding of radiolabeled IFN- $\gamma$  to the mAb (88). Interestingly, this region of IFN- $\gamma$  is predicted to contain an amphipathic  $\alpha$ -helix between positions three and eleven. Amphipathic helices are thought to be involved in immunodominance of antigens related to B-cell and T-cell recognition (96, 97).

The first specific aim of this dissertation is to characterize the epitope specificity of this mAb in more detail. Competition studies with peptide truncations determined that residues 3, 4, and 5 of IFN- $\gamma$  were required for binding to the mAb. These residues are predicted to participate in an amphipathic  $\alpha$ -helix spanning residues 3-11 of IFN- $\gamma$ . The tyrosine at position 14 was also required as its removal in C-terminal truncations caused the loss of blocking ability. It can be concluded that the IFN- $\gamma$  epitope for the neutralizing mAb involves residues 3-14, spanning 12 residues, and it appears that residues 3, 4, 5, and 14 are an important part of the epitope and that this epitope can be considered linear and discontinuous. The data presented here provide further insight to the structure of a site that is involved in receptor interaction.

#### Material and Methods

Synthetic peptides. Peptides were synthesized with a Biosearch 9500AT automated peptide synthesizer using Fmoc chemistry (98). Specific analogs were partially constructed using a manual Fmoc synthesis system (RaMPS, Dupont). Peptides were assembled to a given point in the sequence with the automated synthesizer. The peptide bearing resin was then divided and individual analogs completed with the manual system. Peptides were cleaved at 25° C from the resins using trifluoroacetic acid/phenol/ethanedithiol at a volume ratio

of 95.0/4.5/0.5, respectively. The cleaved peptide was then precipitated in ether and ethyl acetate and subsequently dissolved in water and lyophilized. Reverse phase HPLC analysis of crude peptides indicated one major peak in each profile. Synthetic peptides were submitted to the Protein Core Facility at the University of Florida for amino acid analysis. Figures 2-1 through 2-4 are HPLC profiles and amino acid analyses of the crude peptides used in these studies.

Radioiodination of recombinant IFN- $\gamma$ . Recombinant murine IFN- $\gamma$  was obtained from Schering-Plough Pharmaceutical (Bloomfield, NJ) with a specific activity of  $5 \times 10^5$  units per mg of protein. A unit of IFN- $\gamma$  activity is defined as the concentration of IFN- $\gamma$  that reduces by 50% the plaque formation of vesicular stomatitis virus on mouse L cells (99). IFN- $\gamma$  was labeled with  $^{125}\text{I}$  by using chloramine T as described (87). The specific activity of  $^{125}\text{I}$ -IFN- $\gamma$  was generally 25-35  $\mu\text{Ci}/\mu\text{g}$  protein ( $1\text{Ci} = 37 \text{ GBq}$ ).

MAb to IFN- $\gamma$ . MAb 5.102.12 was previously produced (87) from the fusion of spleen cells from an Armenian hamster immunized with IFN- $\gamma$ . Tissue culture supernatants containing mAb were applied to a protein-A sepharose column. Antibody was eluted with 0.58% acetic acid in 0.15 M NaCl. Eluted fractions were immediately neutralized with one-tenth volume of 1.5 M tris-HCl, pH 8.8, and dialyzed against phosphate

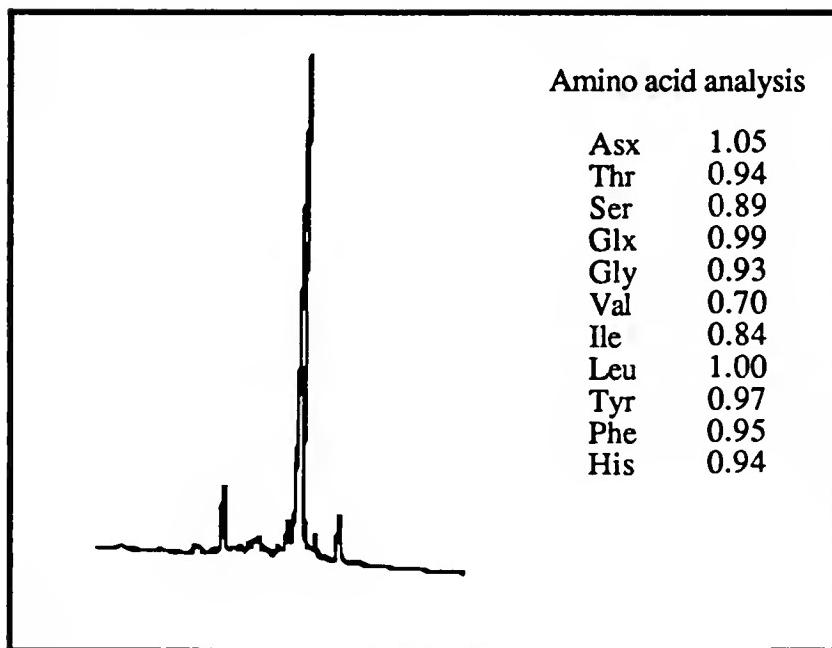


Figure 2-1. HPLC profile and amino acid analysis of IFN- $\gamma$ (1-20). Amino acid analysis values are the ratio of expected number of residues divided by the actual number of residues observed.

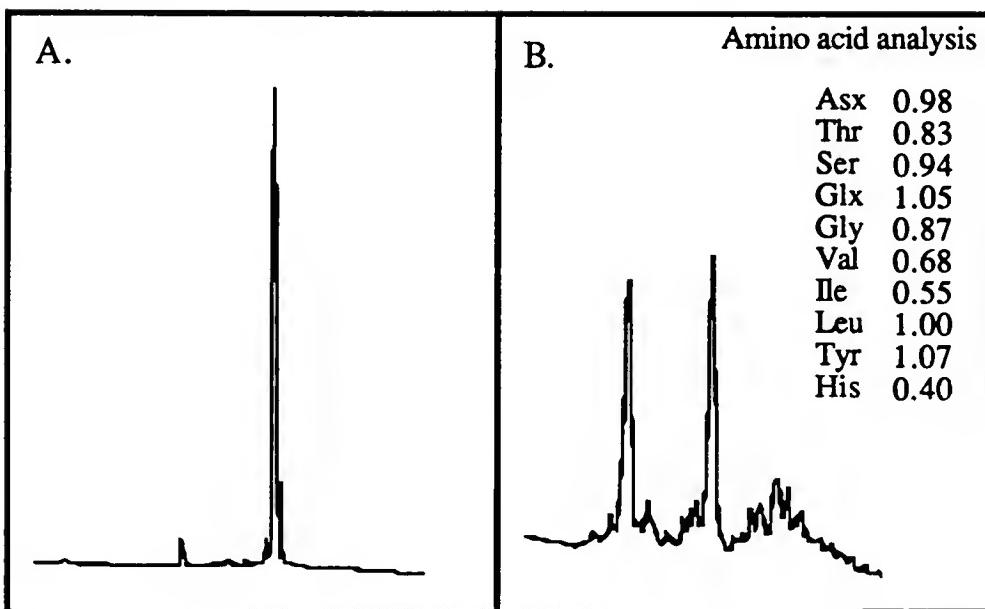


Figure 2-2. Purity data for synthetic peptides. A. IFN $\gamma$ (2-20) HPLC profile. This peptide was synthesized from the same resin as IFN $\gamma$ (1-20) so no amino acid analysis was done. B. IFN $\gamma$ (1-14) HPLC profile and amino acid analysis.

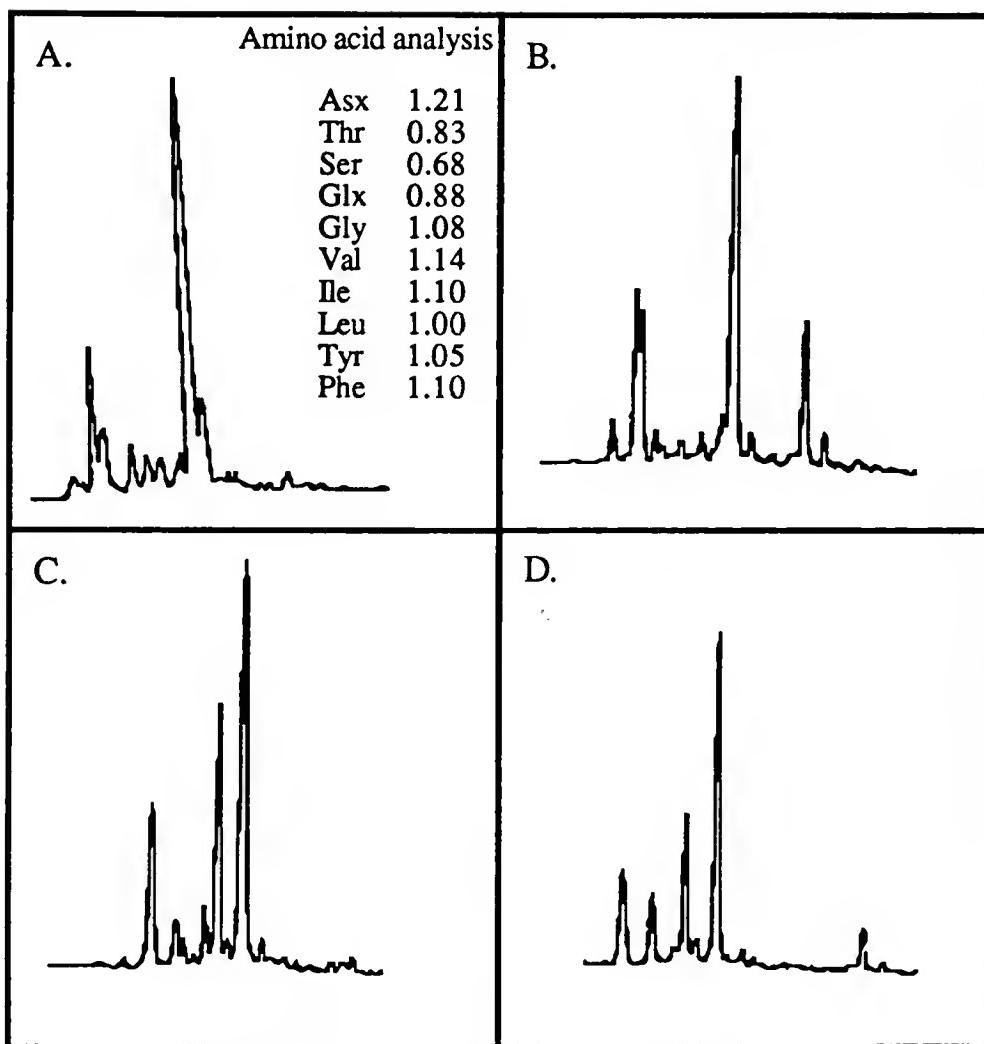


Figure 2-3. Purity information of synthetic peptides of mouse IFN $\gamma$ . A. Amino acid analysis and HPLC profile of IFN $\gamma$ (3-20). B. HPLC profile of IFN $\gamma$ (4-20). C. HPLC profile IFN $\gamma$ (5-20). D. HPLC profile of IFN $\gamma$ (6-20). Amino acid analysis was performed on IFN $\gamma$ (3-20) only because all other peptides were synthesized from the same resin.

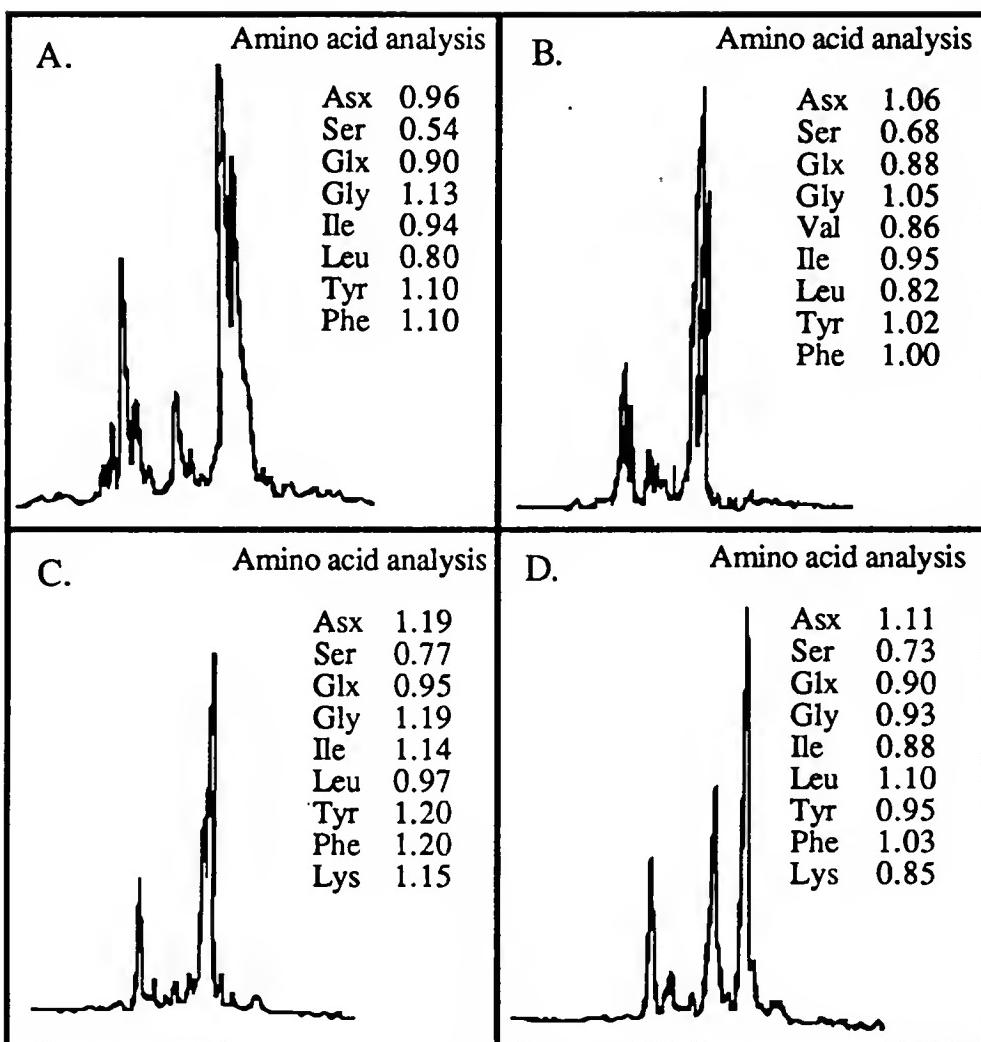


Figure 2-4. Purity information of synthetic peptides of mouse IFN $\gamma$ . A. Amino acid analysis and HPLC profile of IFN $\gamma$ (3-20) S<sup>3</sup>I<sup>4</sup>L<sup>5</sup>. B. Amino acid analysis and HPLC profile of IFN $\gamma$ (3-20) S<sup>3</sup>L<sup>4</sup>V<sup>5</sup>. C. Amino acid analysis and HPLC profile of IFN $\gamma$ (3-20) I<sup>3</sup>K<sup>4</sup>Y<sup>5</sup>. D. Amino acid analysis and HPLC profile of IFN $\gamma$ (3-20) L<sup>3</sup>Y<sup>4</sup>K<sup>5</sup>.

buffered saline, pH 7.4. MAb 5.102.12 is an N-terminal specific mAb raised against IFN- $\gamma$  and has been partially characterized previously (87,88). This mAb has the ability to neutralize the antiviral and macrophage priming activity of IFN- $\gamma$  as well as block the specific binding of  $^{125}\text{I}$ -IFN- $\gamma$  to its cell surface receptor. The N-terminal specificity of the mAb was demonstrated by its binding to a  $^{125}\text{I}$ -labeled synthetic peptide corresponding to the first 39 residues of IFN- $\gamma$ , IFN- $\gamma$ (1-39), and by its ability to block the binding of IFN- $\gamma$  by 5.102.12 in a competitive radioimmunoassay (RIA) (87). The epitope was further localized to the first 20 residues of IFN- $\gamma$ , IFN- $\gamma$ (1-20), using a competitive RIA (88).

Radioimmunoassay (RIA) and ELISA. Assays were performed in duplicate at room temperature as described (100). Briefly, mAb 5.102.12 was added to protein A (Sigma, P6650) coated wells of 96 well PVC microtiter plates (Falcon, 3912). Plates were washed of excess mAb after which 20  $\mu\text{l}$  of  $^{125}\text{I}$ -IFN- $\gamma$  was added. An equal volume of competitor or medium was added thirty minutes prior to the addition of labeled IFN- $\gamma$ . After one hour, wells were washed, cut out and counted on a gamma counter (LKB). Competition was assessed by comparing cpm values to that of medium controls. Competitive ELISA was carried out with the same protocol except biotinylated IFN- $\gamma$  was substituted for the radiolabeled IFN- $\gamma$ . After the mixture was allowed to react for one hour, the plates were washed and alkaline phosphatase conjugated streptavidin (Sigma) was added to the wells. After one hour the plates

were washed and substrate was added. Color development was monitored on a microplate reader (BioRad).

Circular dichroism (CD). CD for selected peptides was determined at room temperature using a JASCO 500C spectropolarimeter. Scans were done with a 0.1 cm pathlength cell at a sensitivity of 0.5-2.0 and a time constant of 8 seconds. The wavelength range measured was from 250 nm to 200 nm at a scan rate of 20 nm/ min. Scans were carried out on peptides in 25% trifluoroethanol (TFE) in water at concentrations of 0.1 to 0.5 mg/ml (101-103). The CD spectra were expressed in terms of  $\Delta\epsilon$  related to the mean residue ellipticity at a given wavelength ( $[\theta]_{\lambda}$ ) for each peptide.

The following formula was used to generate  $\Delta\epsilon$  (104):

$$\Delta\epsilon = \frac{[\theta]_{\lambda}}{3298} \quad [\theta]_{\lambda} = \frac{[\theta]_{\text{observed}}}{c \times l}$$

where  $[\theta]_{\lambda}$  and  $[\theta]_{\text{observed}}$  are expressed in degrees, c equals the mean residue concentration in moles/liter, and l is the pathlength of the cell in cm.

### Results and Discussion

Preliminary studies suggested that the N-terminal residues of IFN- $\gamma$ (1-20) played an important role in the epitope for mAb 5.102.12 (88). In order to ascertain the N-terminal residue requirement for the epitope of this mAb in more detail, I synthesized IFN- $\gamma$ (1-20) and its truncated forms, IFN- $\gamma$ (2-20), IFN- $\gamma$ (3-20), IFN- $\gamma$ (4-20), IFN- $\gamma$ (5-20), and IFN- $\gamma$ (6-20), and tested the peptides for their ability to

block specific binding of  $^{125}\text{I}$ -IFN- $\gamma$  to 5.102.12 in a competitive RIA. The peptides IFN- $\gamma$ (1-20), IFN- $\gamma$ (2-20), and IFN- $\gamma$ (3-20) all had similar ability to block IFN- $\gamma$  binding (Figure 2-5). In addition, as the peptides were shortened they correspondingly lost blocking ability incrementally until they reached position six where IFN- $\gamma$ (6-20) lost blocking function. The third, fourth, and fifth residues appear to be the most important because the peptides lost their blocking ability when these residues were deleted.

The observation that it took at least 1000 times more peptide to block IFN- $\gamma$  binding to mAb illustrates the type of reaction that is occurring in peptide competition studies of this nature. The interaction of a synthetic peptide with a monoclonal antibody raised against a native protein is actually a cross reaction (105). The peptide is not the actual antigen and probably adopts the conformation of the intact protein for only a small percentage of the time. This can account for the lower affinities commonly seen with peptide-antibody interactions.

It had been previously reported that IFN- $\gamma$ (3-20) had a reduced blocking activity compared to IFN- $\gamma$ (1-20) (88). The greater activity of IFN- $\gamma$ (3-20) observed in the present report can possibly be attributed to a change in synthesis chemistry. The peptides in the previous report were synthesized by t-Boc chemistry and cleaved by HF cleavage procedures, whereas the peptides utilized in the present study were synthesized by Fmoc chemistry. The purity data

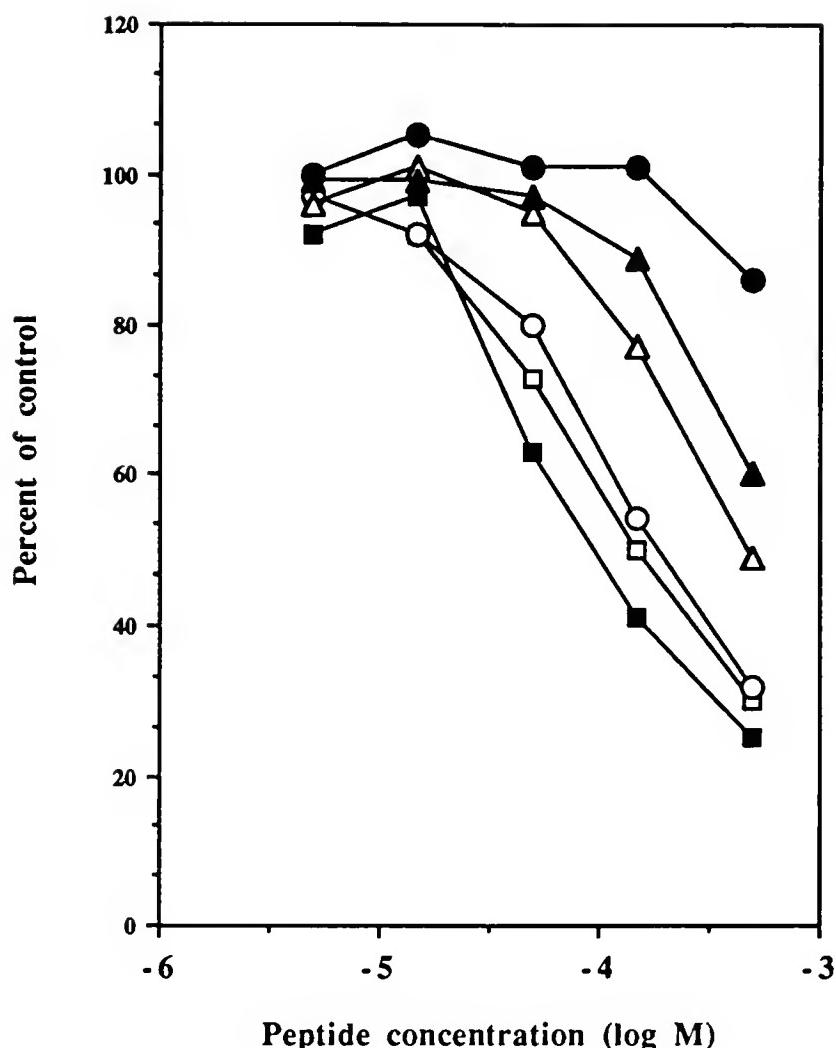


Figure 2-5. Competitive RIA with synthetic peptides of IFN- $\gamma$  and mAb 5.102.12. The ability of synthetic peptides, IFN- $\gamma$ (1-20) and its N-terminal truncations, to block  $^{125}\text{I}$ -IFN- $\gamma$  (20nM) binding to mAb 5.102.12 was assessed. Competition is expressed as percent of control binding to wells that contain no peptide competitors. Control binding was  $33,816 \pm 100$  cpm (cpm  $\pm$  S.D.) in the absence of competition. The figure is representative of three independent experiments. Symbols: IFN- $\gamma$ (1-20) (■), IFN- $\gamma$ (2-20) (□), IFN- $\gamma$ (3-20) (○), IFN- $\gamma$ (4-20) (△), IFN- $\gamma$ (5-20) (▲), IFN- $\gamma$ (6-20) (●).

from the t-Boc synthesis of IFN- $\gamma$ (3-20) was unavailable. In order to illustrate the differences between FMOC and t-Boc synthesis chemistries, I have compared the purity of the peptide IFN- $\gamma$ (1-20) synthesized by both methods. HPLC profiles of IFN- $\gamma$ (1-20)FMOC and IFN- $\gamma$ (1-20)t-Boc are shown in Figure 2-6. The FMOC peptide exists as one major species while the t-Boc peptide is heterogeneous. The multiple peaks seen in HPLC profiles of peptides can contain different components. Some of these peaks are truncations and deletions of the main sequence that are produced during the synthesis. Some peaks are incompletely deprotected peptides that still possess some of the side chain protecting groups that are used in the synthesis. Finally, the remainder of the peaks are peptides that have been chemically modified during the cleavage in hydrogen fluoride which is used for t-Boc cleavage and can be harsh on peptides through the generation of carbonium ions (106). It is likely that the difference seen in the blocking activity of the t-Boc IFN- $\gamma$ (3-20) and the FMOC IFN- $\gamma$ (3-20) is due to modifications of the t-Boc peptide. However, this is only speculation and cannot be determined unequivocally without the original peptide.

One way that residues three to five may function in the epitope for mAb 5.102.12 is that they may actually be a part of the epitope and serve as contact residues for mAb 5.102.12. Another possibility is that they may act to stabilize adjacent contact regions of the epitope. The

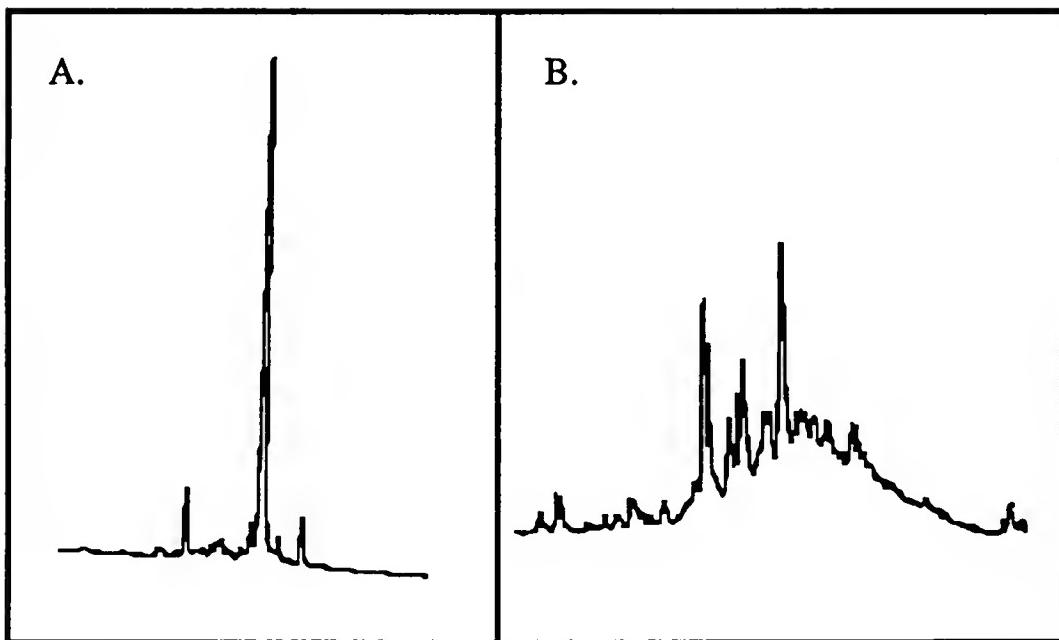


Figure 2-6. HPLC profiles of t-Boc and FMOC peptides. A. IFN- $\gamma$ (1-20) synthesized by FMOC chemistry. B. IFN- $\gamma$ (1-20) synthesized by t-Boc chemistry.

predicted secondary structure of IFN- $\gamma$ (1-20) is presented in Figure 2-7A, and is based on the method of Chou and Fasmen (107,108). The region of 12-20 is proposed to form a loop that may be found on the surface of the molecule and thus have a high probability of forming an antigenic determinant(s) (109). The remainder of the molecule is predicted to be primarily  $\alpha$ -helix. The N-terminal residues three to five, which are depicted as initiating the helix may act to stabilize the helix region thereby stabilizing the main epitope. Interestingly, the predicted helix between residues 3 and 12 is amphipathic in nature (96). Figure 2-7B represents a helix wheel in which the residues found in the predicted helix region are plotted in relation to their positions in the helix. Hydrophilic and hydrophobic residues are separated which is characteristic of an amphipathic helix. The hydrophilic side of the helix presumably interacts with the hydrophilic environment. Amphipathic regions are thought to play a role in immunodominance in B-cell and T-cell recognition (96,97). Amphipathic helices have also been shown to be important in the functional sites of some proteins (110). The amphipathic helix in the C-terminus of IL-2 has been shown to be required for IL-2 function (111).

To gain further insight into the role of the N-terminal residues of IFN- $\gamma$ (1-20) in the epitope, peptide analogs were synthesized with either conservative or nonconservative amino acid substitutions. The criteria for conservative and

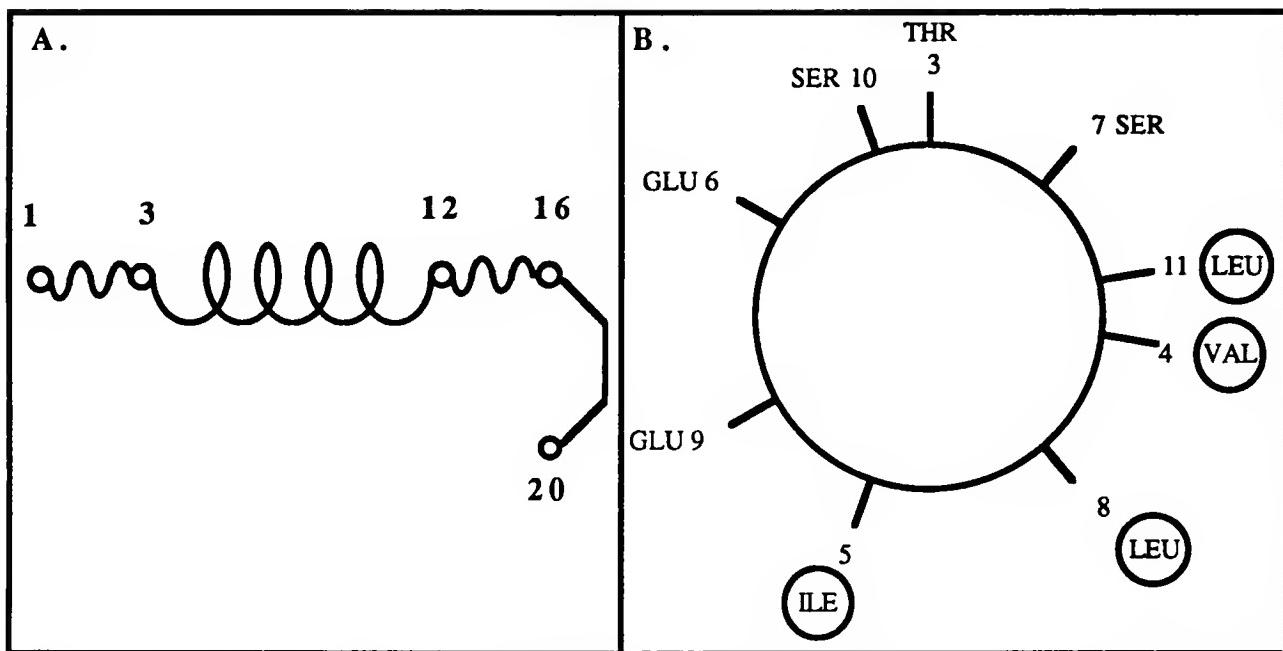


Figure. 2-7. Structure of the N-terminal 20 residues of IFN- $\gamma$ . A. Predicted secondary structure of IFN- $\gamma$ (1-20). This model is derived from the predicted structure of IFN- $\gamma$ (3). Alpha helical structure is depicted as coils, however the number of turns is not intended to represent the actual topography of the region. Wavy lines represent regions that are not predicted to have stable structure (e.g. residues 1-3). The  $\beta$ -bend is represented as a trapezoid shape (residues 16-20). B. Helix wheel depicting amphipathic helix of IFN- $\gamma$  found between residues three and eleven. Hydrophobic residues are circled.

nonconservative substitutions was based on side chain structure, charge, and hydrophobicity (112) of amino acid residues. Due to the incremental nature of the decrease in binding of truncations, positions three, four, and five were changed simultaneously. Table 2-1 presents the native residues as well as analogs of IFN- $\gamma$ (3-20). The native sequence for IFN- $\gamma$ (1-20) at positions three, four, and five consists of threonine, valine, and isoleucine, respectively. Two conservative and two nonconservative analogs were constructed. The first conservative analog contained serine, isoleucine, and leucine at these positions. The second conservative analog had serine, leucine, and valine. These substitutions are similar to the native sequence with respect to charge and side chain structure and are designed to maintain the charge in this region. One nonconservative analog contained isoleucine, lysine, and tyrosine at positions three, four, and five, while the other contained leucine, tyrosine, and lysine. The charge and side chain structure of these substitutions are different than the native residues and are designed to disrupt the charge and hydropathicity in this region. The data presented in Figure 2-8 illustrate the similar ability of the two conservative analogs to block binding of  $^{125}\text{I}$ -IFN- $\gamma$  to 5.102.12 relative to IFN- $\gamma$ (3-20). In contrast, there was a significant reduction in blocking activity by both nonconservative analogs. Thus, the structure of residues three to five of



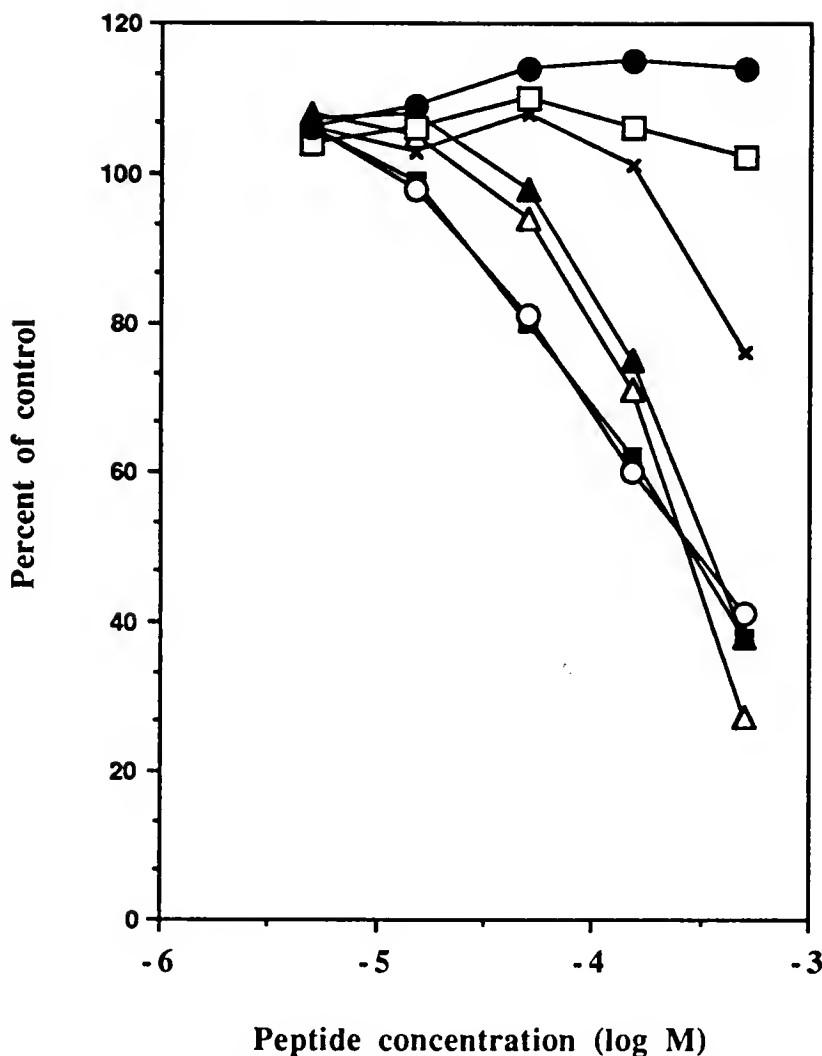


Figure. 2-8. Competitive RIA with peptide analogs of IFN- $\gamma$  and mAb 5.102.12. The ability of IFN- $\gamma$ (1-20), IFN- $\gamma$ (3-20), and their analogs to block  $^{125}$ I-IFN- $\gamma$  (8 nM) binding to mAb 5.102.12 was assessed. Competition is expressed as percent of control binding to wells that contain no peptide competitors. Control binding was  $6947 \pm 451$  cpm (cpm  $\pm$  S.D.). The figure is representative of three independent experiments. Symbols: IFN- $\gamma$ (1-20) (■), IFN- $\gamma$ (3-20) (○), conservative IFN- $\gamma$ (3-20) SER<sup>3</sup>, ILE<sup>4</sup>, LEU<sup>5</sup> (△), nonconservative IFN- $\gamma$ (3-20) SER<sup>3</sup>, LEU<sup>4</sup>, VAL<sup>5</sup> (▲), nonconservative IFN- $\gamma$ (3-20) ILE<sup>3</sup>, LYS<sup>4</sup>, TYR<sup>5</sup> (□), nonconservative IFN- $\gamma$ (3-20) ILE<sup>3</sup>, TYR<sup>4</sup>, LYS<sup>5</sup> (×), IFN- $\gamma$ (6-20) (●).

IFN- $\gamma$  appears to play an important role in the epitope specificity of mAb 5.102.12.

To verify the presence of  $\alpha$ -helical structure in the peptides, CD spectroscopy was employed (104,113). The CD spectra of the peptides are obtained in 25% trifluoroethanol (a secondary structure stabilizing agent) and show the potential of each peptide to form a particular secondary structure. As shown in Figure 2-9, the CD spectra of peptide IFN- $\gamma$ (1-20) indicated mainly  $\alpha$ -helix characterized by a pronounced minimum at 220 nm. IFN- $\gamma$ (3-20) also had helical structure that was less than that of IFN- $\gamma$ (1-20). Conversely, the truncated peptide IFN- $\gamma$ (6-20), which lacked function, lost most of its  $\alpha$ -helical structure. IFN- $\gamma$ (3-20), SER<sup>3</sup>, ILE<sup>4</sup>, LEU<sup>5</sup>, a conservative analog, had  $\alpha$ -helical structure that was slightly less than IFN- $\gamma$ (1-20) but greater than the native truncation IFN- $\gamma$ (3-20). As indicated, this conservative analog was as effective as IFN- $\gamma$ (3-20) in blocking IFN- $\gamma$  binding to mAb 5.102.12. The nonconservative analog, IFN- $\gamma$ (3-20) ILE<sup>3</sup>, LYS<sup>4</sup>, TYR<sup>5</sup>, which lost blocking activity, exhibited almost identical secondary structure to the IFN- $\gamma$ (1-20) (primarily  $\alpha$ -helix). Thus, the loss of blocking activity of substituted analogs was not due to loss of  $\alpha$ -helical structure. Positions three, four, and five apparently participate in the formation and/or stabilization of the  $\alpha$ -helix, based on the lack of helix found in the truncation IFN- $\gamma$ (6-20). The interesting observation that the nonconservative analog did not block function but still

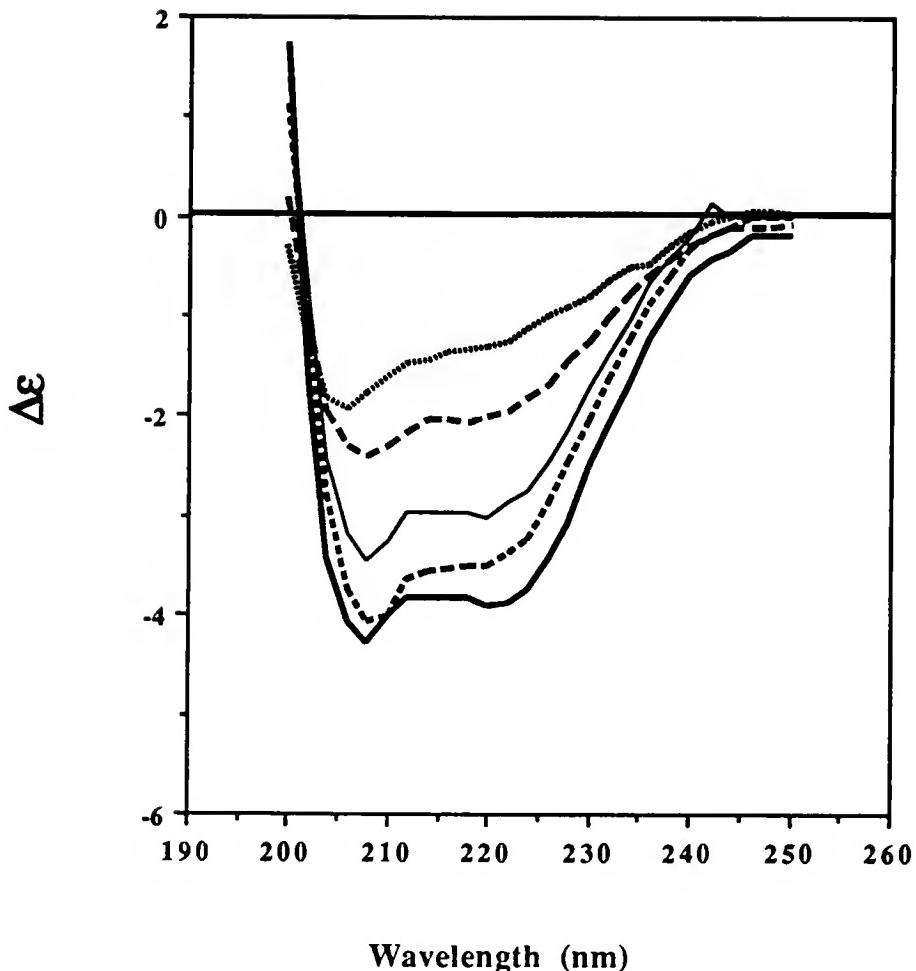


Figure. 2-9. CD spectra of IFN- $\gamma$ (1-20), its analogs, and N-terminal truncations. Spectra were obtained with peptides in 25% TFE as described in the Materials and Methods. Symbols: IFN- $\gamma$ (1-20) (—), IFN- $\gamma$ (3-20) (- - -), IFN- $\gamma$ (6-20) (.....), Conservative analog IFN- $\gamma$ (3-20) SER<sup>3</sup>, ILE<sup>4</sup>, LEU<sup>5</sup> (— · —), Nonconservative analog IFN- $\gamma$ (3-20) ILE<sup>3</sup>, LYS<sup>4</sup>, TYR<sup>5</sup> (- - - -).

retained stable secondary structure would suggest that the  $\alpha$ -helix alone is not critical and that residues three to five are directly involved in the epitope of the N-terminal peptide. The nonconservative substitutions are predicted to participate in  $\alpha$ -helix formation as effectively as the conservative substitutions which is consistent with the stable secondary structure of the analog containing these residues (107). Additionally, the conservative analogs examined maintain the amphipathic nature of the helix, while the nonconservative analogs disrupt it through changes in hydrophobicity and charge. Thus, the structural and functional data implicate the third, fourth, and fifth residues directly in the epitope of mAb 5.102.12, and that the charge and hydropathicity of these residues is important.

Peptides were synthesized with C-terminal truncations in order to determine the role of residues in the putative loop region in the epitope specificity of mAb 5.102.12.

IFN- $\gamma$ (1-14) and IFN- $\gamma$ (1-20) had similar blocking ability, whereas IFN- $\gamma$ (1-13) possessed no activity, which is evidence that tyrosine at position 14 is important for epitope structure (figure 2-10). IFN- $\gamma$ (3-14) also had full blocking activity. Thus, the minimum length requirement of the epitope for mAb 5.102.12 involves residues 3 to 14.

I next examined the secondary structure of C-terminal truncations of IFN- $\gamma$ (1-20). Both IFN- $\gamma$ (1-14) and IFN- $\gamma$ (1-13) had an apparent reduced helical structure, but not complete abrogation, with an apparent increase in random structure

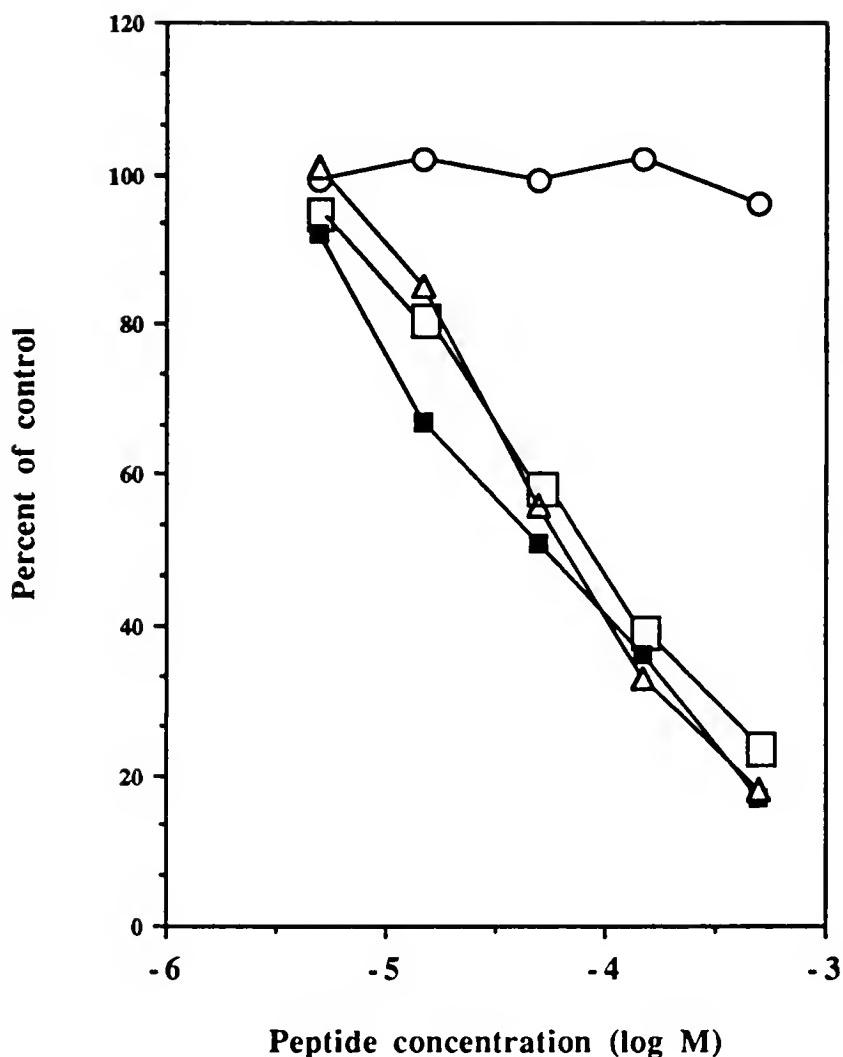


Figure. 2-10. Competitive RIA with C-terminal truncations of IFN- $\gamma$ (1-20) and mAb 5.102.12. The ability of IFN- $\gamma$ (1-20) and its C-terminal truncations to block  $^{125}\text{I}$ -IFN- $\gamma$  (22 nM) binding to mAb 5.102.12 was assessed. Competition is expressed as percent of control binding to wells that contain no peptide competitors. Control binding was  $56,204 \pm 153$  cpm (cpm  $\pm$  S.D.). The figure is representative of three independent experiments. Symbols: IFN- $\gamma$ (1-20) (■), IFN- $\gamma$ (1-13) (○), IFN- $\gamma$ (1-14) (Δ), IFN- $\gamma$ (3-14) (□).

(minimum at 205-200 nm) (figure 2-11). The reduction in  $\alpha$ -helix in these truncations may involve a partial loss of helix in the C-terminal region of the peptide caused by the removal of residues C-terminal to the helix (residues 3-11). Since both peptides had similar reduction in helix and IFN- $\gamma$ (1-14) but not IFN- $\gamma$ (1-13) blocked mAb binding, it suggests that factors additional to  $\alpha$ -helix may play a role in the epitope.

All of the experiments described have used crude peptides. Although, as I have shown, the purity of FMOC peptides is higher than t-Boc, the differences in purity as shown by HPLC profiles of the peptides used warrants further examination. To attempt to address this issue, two peptides, IFN- $\gamma$ (1-20) and IFN- $\gamma$ (1-13) were purified by HPLC. IFN- $\gamma$ (1-20) had a final purity of 90% and IFN- $\gamma$ (1-13) had a final purity of 96% as determined by HPLC. Amino acid analysis and HPLC profiles of the purified are shown in figure 2-12. The results of a competitive ELISA (Figure 2-13) show that crude and purified IFN- $\gamma$ (1-20) have identical blocking profiles. Purified IFN- $\gamma$ (1-13) has no blocking activity. Therefore, no impurity was responsible for blocking activity. Additionally, the lack of purity was not responsible for a lack of blocking activity.

To further explore this issue, I will attempt to explain the nature of the impurities found in crude synthetic peptides and relate this to my findings. Much of the information that I present here comes from my own

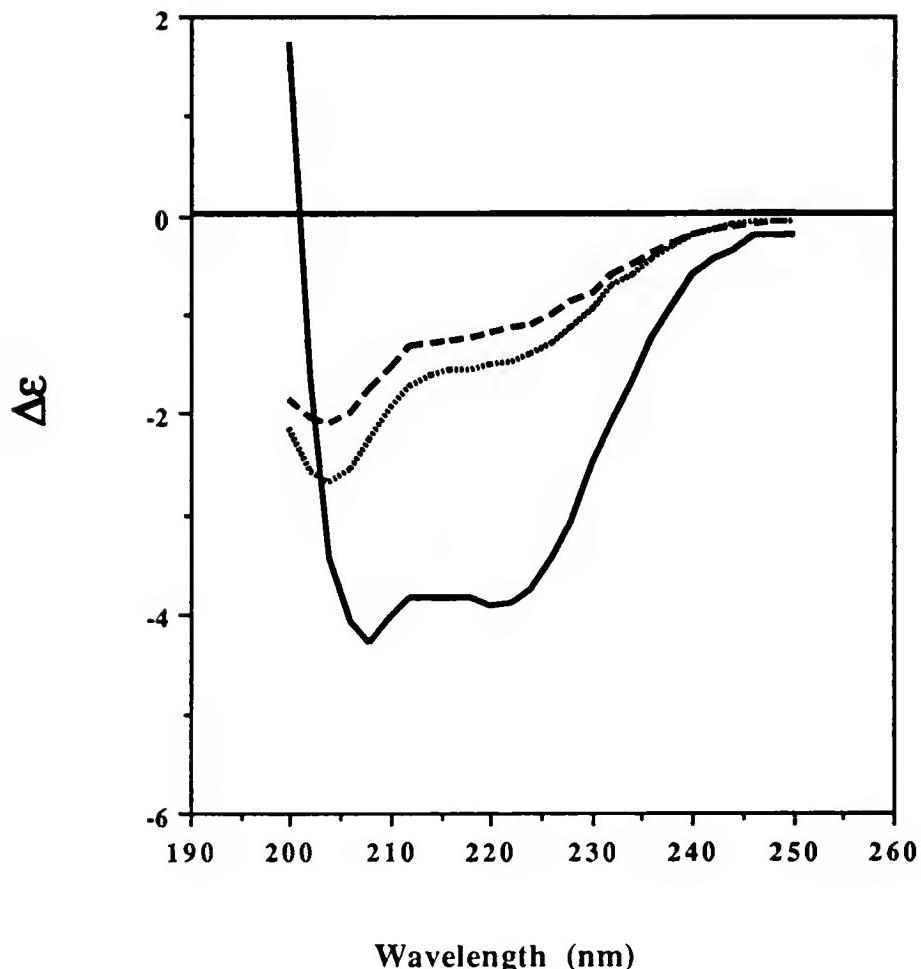


Figure 2-11. CD spectra of IFN- $\gamma$ (1-20) and its C-terminal truncations. Spectra were obtained with peptides in 25% TFE as described in the Materials and Methods. Symbols:  
IFN- $\gamma$ (1-20) (—), IFN- $\gamma$ (1-13) (-----),  
IFN- $\gamma$ (1-14) (- - -).

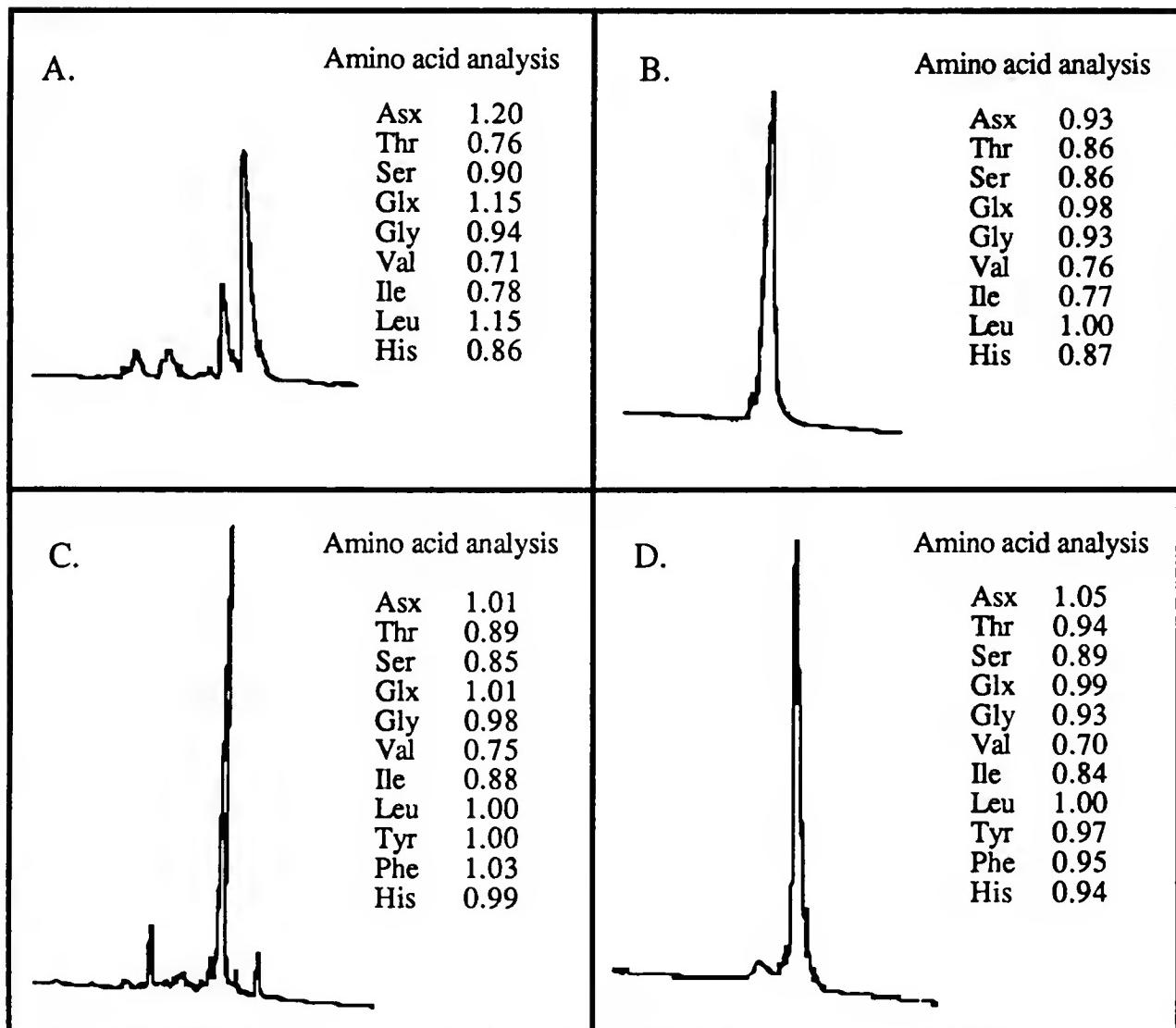


Figure 2-12. Amino acid analysis and HPLC profiles on purified synthetic peptides. Amino acid analysis values are the ratio of expected number of residues divided by the actual number of residues observed. A. IFN- $\gamma$ (1-13) crude. B. IFN- $\gamma$ (1-13) purified. C. IFN- $\gamma$ (1-20) crude. D. IFN- $\gamma$ (1-20) purified.

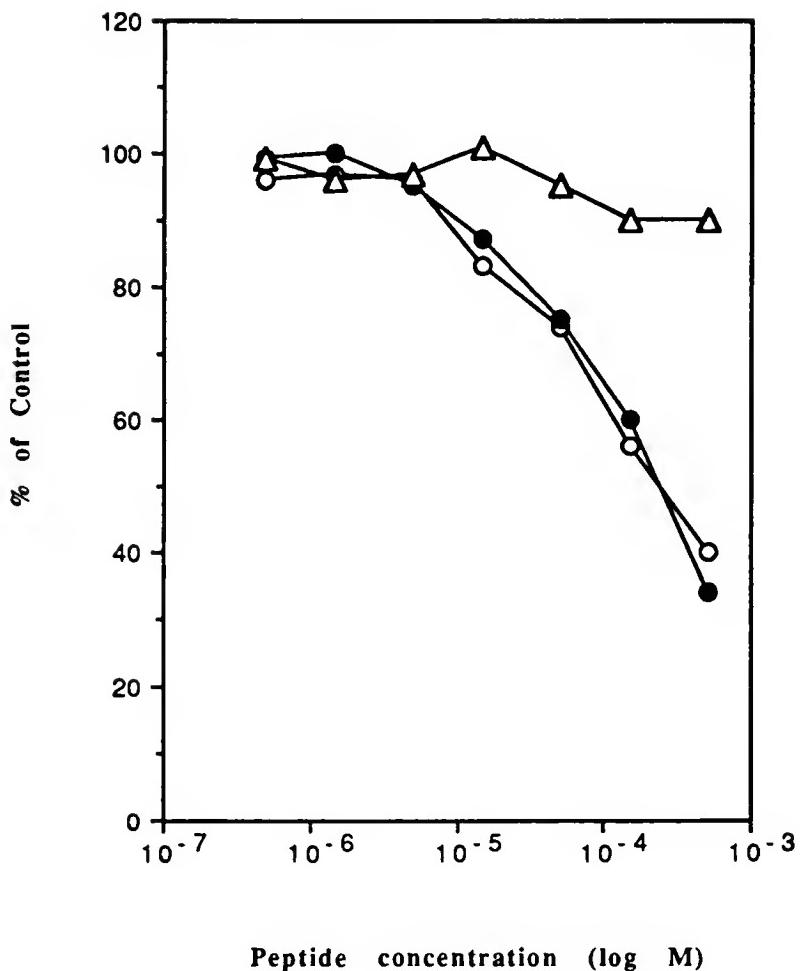


Figure 2-13. Competitive ELISA of purified synthetic peptides of IFN- $\gamma$ . ELISA was carried out under the conditions described in materials and methods. Biotinylated IFN- $\gamma$  concentration used was 25 nm. Symbols: IFN- $\gamma$ (1-20) crude (●), IFN- $\gamma$ (1-20) purified (○), IFN- $\gamma$ (1-13) purified (Δ).

observations and from discussion with peptide chemists at Biosearch and Advanced Chemtech. Most of the impurities are due to amino acid deletions and/or the incomplete removal of side chain protecting groups. Amino acid deletions can be identified by amino acid analysis. Incomplete deprotection is commonly seen with the MTR group on arginine and the TRT group on histidine. However, other protecting groups can also be difficult to remove, depending on the sequence of the peptide. Frequently these "impure" peptides, due to the nature of the assay being used, exhibit the same activity as complete sequence. This may be because some of the residues affected may not be important for the activity of the peptide.

It has been my observation that HPLC alone cannot give a clear picture as to the purity of a synthetic peptide. Protein sequencing is the only certain way of determining, beyond question, the content of a peptide. As this is impractical in routine situations, amino acid analysis must be used to verify the presence of each amino acid residue. When interpreting an amino acid analysis, the ratio of observed to expected should be close to  $1.0 \pm 10\%$ , with some exceptions. Serine, valine, methionine, isoleucine, histidine, and tyrosine can be found in lower amounts due to modifications during the hydrolysis reaction for the amino acid analysis. Tryptophan and cysteine require special hydrolysis. In my opinion, each peptide presented here has

an acceptable amino acid analysis and all of the residues are present in reasonable quantities.

Differences in synthesis can be responsible for varied purity of peptides. Each synthesis is unique to the sequence being synthesized. Although two sequences are similar one sequence may be more easily synthesized than the other.

Another observation that I have made concerns the content of peaks seen in HPLC profiles. Due to the high absorption seen with some protecting groups used in the synthesis, a relatively large peak can contain a disproportionately small amount (by weight) of material when isolated and analyzed. The large peak observed can be misleading.

A further indication of the suitability of crude peptides in these mapping studies can be illustrated with the following examples. Blocking studies presented in this dissertation are interpreted in a qualitative manner. A peptide either blocks or does not block IFN- $\gamma$  binding to mAb. One exception to this involves the N-terminal truncations of IFN- $\gamma$ (1-20) (IFN- $\gamma$ (3-20), IFN- $\gamma$ (4-20), IFN- $\gamma$ (5-20), and IFN- $\gamma$ (6-20)). These peptides lose blocking activity incrementally as residues are left off. If one examines the HPLC profiles of IFN- $\gamma$ (5-20) and IFN- $\gamma$ (6-20) (Figure 2-3 C,D) they appear to be qualitatively similar. Also, these peptides were synthesized from the same resin. However, when ILE at position 5 is left off as in IFN- $\gamma$ (6-20) blocking activity is reduced (Figure 2-5). This would suggest that

the lack of ILE at position 5 is responsible for this difference in blocking activity, and not a difference in purity. The difference seen in activity appears to be greater than a difference in the HPLC profiles of the peptides. The peptide IFN- $\gamma$ (1-14) has blocking activity similar to IFN- $\gamma$ (1-20) as shown in figure 2-10. The amino acid analysis appears to be reasonable based on the sequence (Figure 2-2). It shows the presence of TYR in the peptide while the amino acid analysis of IFN- $\gamma$ (1-13) does not (Figure 2-12). The HPLC profile contains two major peaks, each about 30-40% of the total. The content of the second peak is not known but it is likely to be an incompletely deprotected peptide. This impurity seems to not have an effect on blocking activity. Also, if the peptide were three fold greater in purity (>90% pure), one would expect little difference in blocking activity. The profile for IFN- $\gamma$ (1-14) would be shifted slightly to the left one half log and not be very different than the profile of IFN- $\gamma$ (1-20). The lack of TYR in IFN- $\gamma$ (1-13) seems to be what is responsible for a lack of blocking activity.

The data presented in this chapter indicate that the epitope specificity of the IFN- $\gamma$  neutralizing mAb 5.102.12 spans 12 amino acid residues, and is located between the third and fourteenth residues of IFN- $\gamma$  with residues 3, 4, 5, and 14 critical to the interaction of mAb 5.102.12 with IFN- $\gamma$ . Epitopes are classified into four groups, linear, conformational, continuous, and discontinuous (105).

Discontinuous and conformational epitopes are those that are made up of more than one distant site on the surface of a protein. The contact residues are separated by non-critical residues (105). A classic conformational epitope that has been studied is in the molecule lysozyme. X-ray crystallographic studies of a monoclonal antibody binding to an epitope on lysozyme have detailed a conformational epitope. Linear and continuous epitopes are epitopes found on short sequences not more than eight to ten residues in length that involve a single stretch of residues (105,114-118). Synthetic peptides are common continuous and linear epitopes. This epitope for 5.102.12 does not appear to be continuous in the classical sense based on its length, twelve residues. It also does not appear to require a stable conformation based on CD studies. Based on the requirement of the residues 3,4,5, and 14, the linear peptide IFN- $\gamma$ (3-14) appears to contain a discontinuous epitope. The role that internal residues may play in this epitope is as yet undetermined. Thus, the data suggest that the mAb binding site on IFN- $\gamma$  exists as a linear discontinuous epitope. The classification of linear discontinuous epitope has been used in another report (119). In this case the terminology was discontinuous linear epitope and was used to describe the epitope specificities of several mAb to synthetic peptides.

CHAPTER 3  
TOPOLOGY OF RECEPTOR BINDING DOMAINS OF MOUSE IFN- $\gamma$  THAT  
INCLUDE THE N-AND C-TERMINI.

Introduction

IFN- $\gamma$  possesses a number of immunologic activities that are critical to immune function. The identification of the receptor binding domains should further enhance the understanding of its mechanism of action. This information would be useful for the design of agonists and antagonists of IFN- $\gamma$  function. The location of these domains is unclear. The N-terminus of IFN- $\gamma$  has been shown to bind to the receptor as determined by competition studies where the first 39 residues of IFN- $\gamma$ , IFN- $\gamma$ (1-39), compete with IFN- $\gamma$  for its receptor (88). Antibody studies confirm the role of the N-terminus of IFN- $\gamma$  in receptor binding (87,88). The epitope specificity of an IFN- $\gamma$  neutralizing monoclonal antibody was mapped to the first 14 residues of IFN- $\gamma$  (Chapter 2). The binding site on IFN- $\gamma$  for receptor probably involves regions in addition to IFN- $\gamma$ (1-39), since this peptide binds to receptor at 1/1000th the affinity of IFN- $\gamma$  (88). The functional significance of the remainder of the molecule has not been determined. Additionally, the role of the C-terminus in IFN- $\gamma$  function has been controversial. A

truncated form of recombinant human IFN- $\gamma$  missing the C-terminal 23 residues was shown to possess full activity (82). In contrast, the removal of 11 residues from the C-terminus of IFN- $\gamma$ , with limited proteolysis, yielded IFN- $\gamma$  with significantly reduced activity (81). Studies have shown that polyclonal antibodies directed to a C-terminal synthetic peptide, IFN- $\gamma$ (95-133), neutralized IFN- $\gamma$  activity (87). Similarly, mAb that appear to be directed to the C-terminus of IFN- $\gamma$  also neutralize IFN- $\gamma$  activity (84).

The second specific aim of this dissertation is to explore other regions of the molecule for functional significance using long overlapping synthetic peptides encompassing the entire sequence of IFN- $\gamma$  and antiserum raised to these peptides. The data strongly suggests that the C-terminus, in addition to the N-terminus, of mouse IFN- $\gamma$  is important in receptor binding and function. Using synthetic peptides, antisera raised against these peptides, circular dichroism spectra for secondary structure, and predictive algorithms for secondary and tertiary structure, a three dimensional model of IFN- $\gamma$  has been constructed that includes both the N and C-termini as receptor binding domains.

#### Materials and Methods

Animals and immunizations. Female New Zealand White rabbits (2-2.5kg) were immunized with synthetic peptides

conjugated with keyhole-limpet hemocyanin using gluteraldehyde conjugation (120). One hundred micrograms of conjugated peptide were injected with Complete Freund's adjuvant in a total volume of 0.5 ml followed 30 days later with 100 µg of conjugated peptide in Incomplete Freund's adjuvant. Seven days later blood was drawn and serum was collected. Subsequent boost injections occurred at 30 day intervals followed by bleeds seven days later.

Synthetic peptides. Peptides were synthesized with a Biosearch 9500AT automated peptide synthesizer using Fmoc chemistry (98). Peptides were cleaved at 25° C from the resins using trifluoroacetic acid/phenol/ethanedithiol at a volume ratio of 95.0/4.5/0.5. The cleaved peptides were then precipitated in ether and ethyl acetate and subsequently dissolved in water and lyophilized. The purity of peptides was assessed by reverse phase HPLC and verified by amino acid analysis. Amino acid analysis and HPLC profiles for the peptides used for immunization are found in figure 3-1. The peptides used in epitope mapping studies were purified using a preparative reverse phase HPLC column (Vydac). The major peak was collected, lyophilized, and checked for purity with analytical HPLC and amino acid analysis. The amino acid analysis and HPLC profiles for purified peptides are found in figure 3-2.

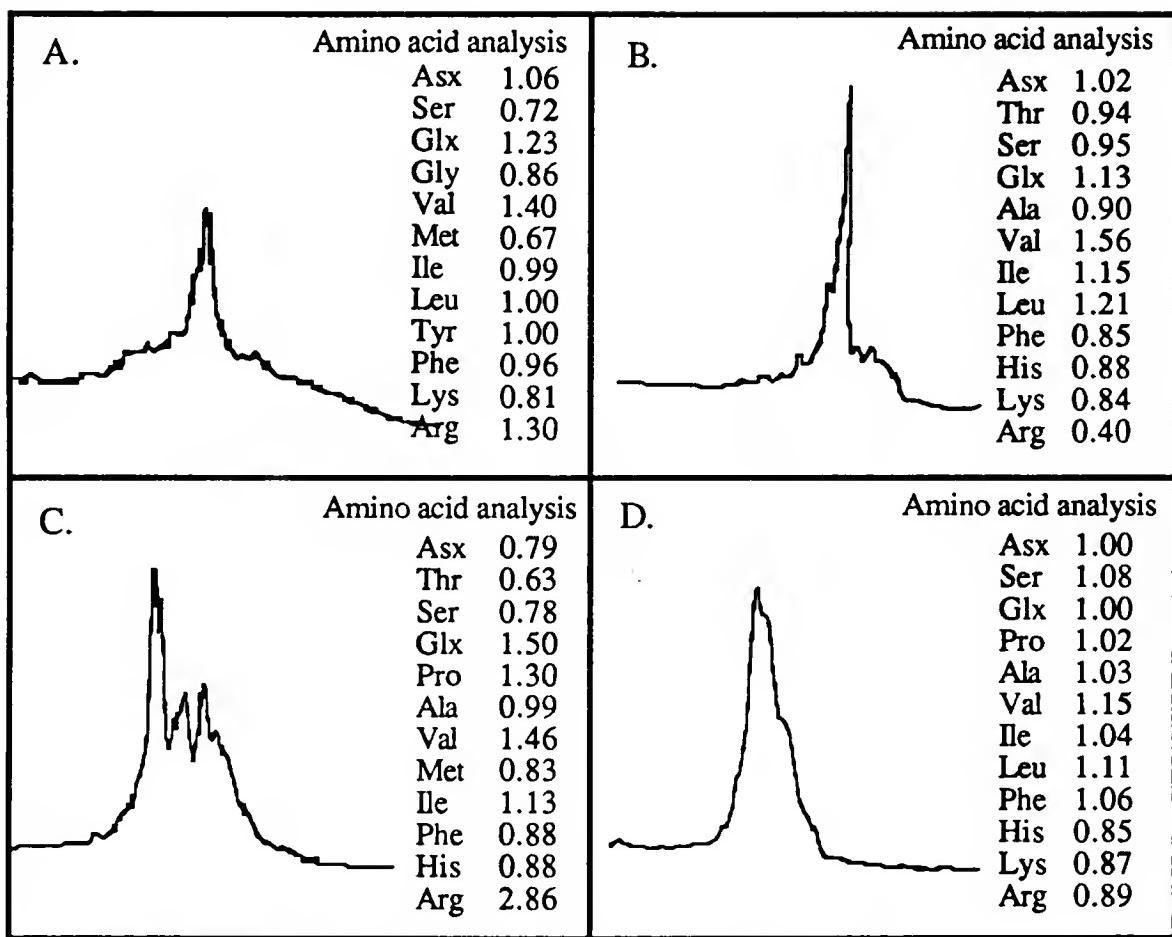


Figure 3-1. Purity information of overlapping synthetic peptides of mouse IFN- $\gamma$ . A. IFN- $\gamma$ (36-60). B. IFN- $\gamma$ (54-91). C. IFN- $\gamma$ (78-107). D. IFN- $\gamma$ (95-133).

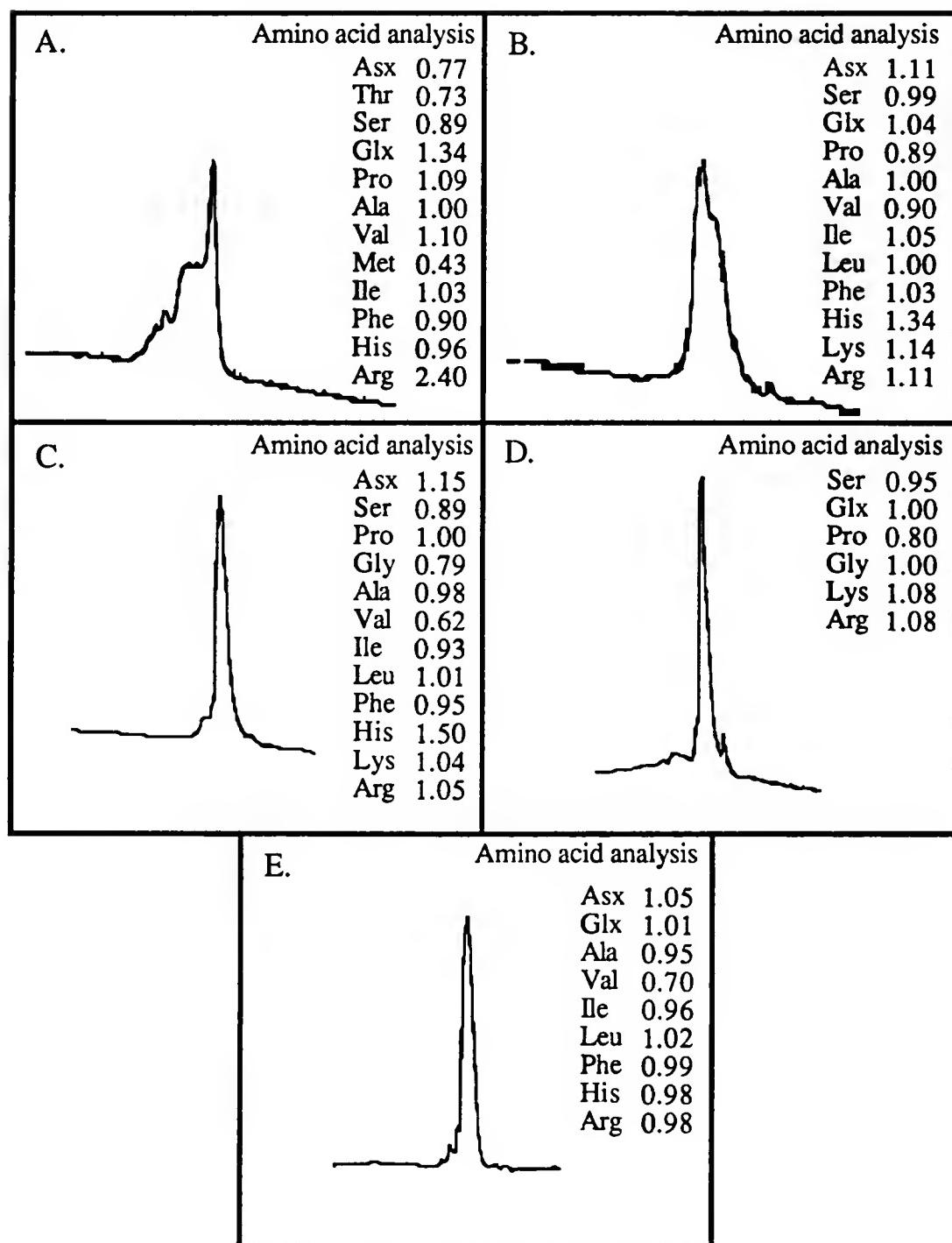


Figure 3-2. Purity information on purified synthetic peptides used in C-terminal specific antibody mapping studies. A. IFN- $\gamma$ (78-107). B. IFN- $\gamma$ (95-133). C. IFN- $\gamma$ (108-133). D. IFN- $\gamma$ (120-133). E. IFN- $\gamma$ (108-119).

ELISA. Synthetic peptides and IFN- $\gamma$  were dissolved in 0.1 M sodium carbonate buffer, pH 9.6, at a concentration of 12  $\mu$ g/ml and 50  $\mu$ l of solution were added to each well of a 96 well flat bottom tissue culture plate (Falcon). After overnight incubation at room temperature, each plate was dried with mild heat applied by a standard blow dryer. Nonspecific binding sites were blocked with 200  $\mu$ l/well 5% Carnation powdered milk dissolved in PBS for 2 hours. Plates were then washed with 0.15 M NaCl/0.05% Tween 20 five times. Dilutions of antisera were added in 50  $\mu$ l amounts and incubated at room temperature for 2 hours. After washing, horseradish peroxidase conjugated goat anti-rabbit IgG antibodies (Cappel) were added to each well and incubated for one hour. The plates were washed and O-phenylenediamine (Sigma) and H<sub>2</sub>O<sub>2</sub> were added and color development was allowed to occur for 15-30 minute after which 25  $\mu$ l of H<sub>2</sub>SO<sub>4</sub> was added to stop the reaction. Absorbance was determined at 492 nm in an ELISA plate reader (BioRad). Endpoints were defined as the highest dilution of antisera with absorbance readings that were twice those of the blank.

Circular dichroism (CD). CD for the peptides was determined as described in chapter 2 except for the following changes. Scans were done with a 0.01 cm pathlength cell at a sensitivity of 1.0 and a time constant of 8 seconds. The wavelength range measured was from 260 nm to 184 nm at a scan rate of 5 nm/ min.

IFN- $\gamma$  neutralization assay. Varying concentrations of IFN- $\gamma$  were incubated with dilutions of rabbit antisera for 30 min at 37°C. Residual IFN- $\gamma$  activity was then measured as described (99). Briefly, IFN- $\gamma$  samples in the presence or absence of antibodies were incubated with mouse L cells for 16 to 18 hr at 37°C after which inhibition of virus replication was determined in a plaque reduction assay with vesicular stomatitis virus. One unit of IFN- $\gamma$  was defined as that which caused a 50% reduction in plaque formation. For epitope mapping studies, synthetic peptides were incubated with antibodies for 30 min at 37°C prior to their addition to IFN- $\gamma$ . The peptide inhibition of IFN- $\gamma$  neutralization by anti-peptide antisera was compared to IFN- $\gamma$  alone and to antibody plus IFN- $\gamma$  in the absence of peptide. For peptide blocking studies, synthetic peptides were substituted for the antibodies. Peptides were dissolved in HMEM containing 2% FBS. No preincubation with IFN- $\gamma$  was performed.

#### Results and Discussion

To examine the role of the remainder of IFN- $\gamma$ , long overlapping peptides were synthesized covering the entire 133 amino acids of IFN- $\gamma$ . The peptides synthesized were: IFN- $\gamma$ (36-60), IFN- $\gamma$ (54-91), IFN- $\gamma$ (78-107), and IFN- $\gamma$ (95-133) and are shown in Table 3-1. These peptides were tested for their abilities to inhibit IFN- $\gamma$  antiviral activity. N-terminal peptide, IFN- $\gamma$ (1-39), had the ability to directly inhibit

Table 3-1. Hydropathicity of IFN- $\gamma$  overlapping synthetic peptides.

| Peptide                | Sequence                                 | Hydropathy <sup>a</sup> |
|------------------------|--|-------------------------|
| IFN- $\gamma$ (1-39)   | HGTVIESTLESLNYYFNSSGIDVEEKSLFLDIWRNWQKDG | -0.559                  |
| IFN- $\gamma$ (36-60)  | QKDGDMDKILQSQIISFYLRLFEVLK               | -0.036                  |
| IFN- $\gamma$ (54-91)  | RLFEVLKDNQAISNNISVIESHLITFFSNSKAKKDAF    | -0.189                  |
| IFN- $\gamma$ (78-107) | TTFFSNSKAKKDAFMSIAKFEVNNPQVQRQ           | -0.617                  |
| IFN- $\gamma$ (95-133) | AKFEVNNPQVQRQAFNELIRVVHQLLPESSLRKRKRSRC  | -0.792                  |

<sup>a</sup> Average hydropathy values were calculated by taking the sum of the hydropathy values for each amino acid divided by the number of amino acids in each sequence. Hydropathy values were taken from Kyte and Doolittle (112).

IFN- $\gamma$  function as shown previously (88) while the remainder of the peptides had no effect (Table 3-2). However, based on competition studies, IFN- $\gamma$ (1-39) interacted with receptor with 1/1000 the affinity of IFN- $\gamma$  (88). Thus, other regions of the IFN- $\gamma$  molecule are likely to be involved in receptor interaction. To determine the role of these other regions of IFN- $\gamma$ , rabbit antibodies were produced to the overlapping synthetic peptides and tested for their abilities to interact with and inhibit IFN- $\gamma$  function. Antisera to IFN- $\gamma$ (1-39) and IFN- $\gamma$ (95-133) had been produced previously (87). These antibodies to IFN- $\gamma$ (1-39) and IFN- $\gamma$ (95-133) were shown to neutralize IFN- $\gamma$  activity by blocking IFN- $\gamma$  receptor binding (87). All of the peptides elicited antibodies reactive with IFN- $\gamma$  as determined by ELISA (Table 3-3). Antibodies directed against N-terminal peptide, IFN- $\gamma$ (1-39), also bound and neutralized IFN- $\gamma$  as shown previously (87). Similarly, antiserum to the C-terminus had the ability to neutralize IFN- $\gamma$  antiviral activity. In contrast, antisera to the internal three peptides had no effect although they bound IFN- $\gamma$ . The differential neutralizing ability was probably not due to the differences in titer. When the neutralization titer is normalized on the basis of ELISA titer to IFN- $\gamma$  by taking the ratio of neutralizing titer to ELISA titer, the ratios were very different. If elevated titer alone was responsible for neutralization then similar ratios would be expected. Additionally, the ELISA titer to anti-IFN- $\gamma$ (78-107) is similar to the titers of the

Table 3-2. Direct peptide blocking of mouse IFN- $\gamma$  antiviral activity by IFN- $\gamma$  synthetic peptides.

| Peptides               | Peptide concentration <sup>a</sup> |
|------------------------|------------------------------------|
| IFN- $\gamma$ (1-39)   | 15 $\mu$ M                         |
| IFN- $\gamma$ (36-60)  | >500 $\mu$ M                       |
| IFN- $\gamma$ (54-91)  | >500 $\mu$ M                       |
| IFN- $\gamma$ (78-107) | >500 $\mu$ M                       |
| IFN- $\gamma$ (95-133) | >500 $\mu$ M                       |

<sup>a</sup>Highest peptide concentration that will block 10 units of IFN- $\gamma$  antiviral activity.

**Table 3-3. IFN- $\gamma$  neutralization efficiency of antipeptide antisera.**

| <u>Peptide</u>                         | <u>Peptide</u>   | <u>ELISA titer (E)<sup>a</sup></u> | <u>Neutralization (N)</u> | <u>Ratio<sup>b</sup></u> |
|--|------------------|------------------------------------|---------------------------|--------------------------|
|  |                  | IFN- $\gamma$                      | against 10U IFN- $\gamma$ | E/N (x100)               |
| IFN- $\gamma$ (1-39)                   | ND               | 1/10,000                           | 1/3000                    | 30.0                     |
| IFN- $\gamma$ (36-60)                  | 1/1000           | 1/1000                             | <1/3                      | <0.3                     |
| IFN- $\gamma$ (54-91)                  | 1/1000           | 1/600                              | <1/3                      | <0.05                    |
| IFN- $\gamma$ (78-107)                 | 1/100,000        | 1/20,000                           | <1/3                      | <0.015                   |
| <u>IFN-<math>\gamma</math>(95-133)</u> | <u>1/300,000</u> | <u>1/60,000</u>                    | <u>1/6000</u>             | <u>10.0</u>              |

<sup>a</sup>ELISA performed as described in materials and methods. Six hundred nanograms of each peptide or IFN- $\gamma$  was used in each well.

<sup>b</sup>Ratio calculated as ELISA titer to IFN- $\gamma$  divided by neutralization titer multiplied by 100.

<sup>c</sup>ND indicates not done.

N- and C-terminal antisera, but does not possess any neutralizing activity. The results confirm the role of the N-terminus as a receptor interaction site and suggest that the C-terminus forms an additional binding domain.

The N-terminal binding domain has been characterized previously using direct peptide competition and epitope mapping of an N-terminal specific IFN- $\gamma$  neutralizing mAb (88, Chapter 2). I was therefore interested in localizing the epitope to which the C-terminal neutralizing antibodies bind thereby further characterizing this site on the molecule. The antiserum, anti-IFN- $\gamma$ (95-133), is polyclonal and potentially contains antibodies to several epitopes, so there is the possibility that only a subset of these antibodies is responsible for the neutralizing activity seen in this antiserum. Peptide truncations of IFN- $\gamma$ (95-133) were synthesized and used to map its neutralizing epitope(s). Figure 3-3 illustrates the peptides synthesized in these experiments and their relationship to one another. To map the neutralizing epitope(s) of anti-IFN- $\gamma$ (95-133), a modification of the antibody neutralization assay was used in which synthetic peptides were added to inhibit the IFN- $\gamma$  neutralization by anti-IFN- $\gamma$ (95-133). The immunizing peptide, IFN- $\gamma$ (95-133), was able to completely inhibit the neutralizing activity of anti-IFN- $\gamma$ (95-133) (Table 3-4). A truncation, IFN- $\gamma$ (108-133), was able to inhibit neutralization about 30% at the same concentration. IFN- $\gamma$ (78-107) as well as the shorter peptides IFN- $\gamma$ (120-133)

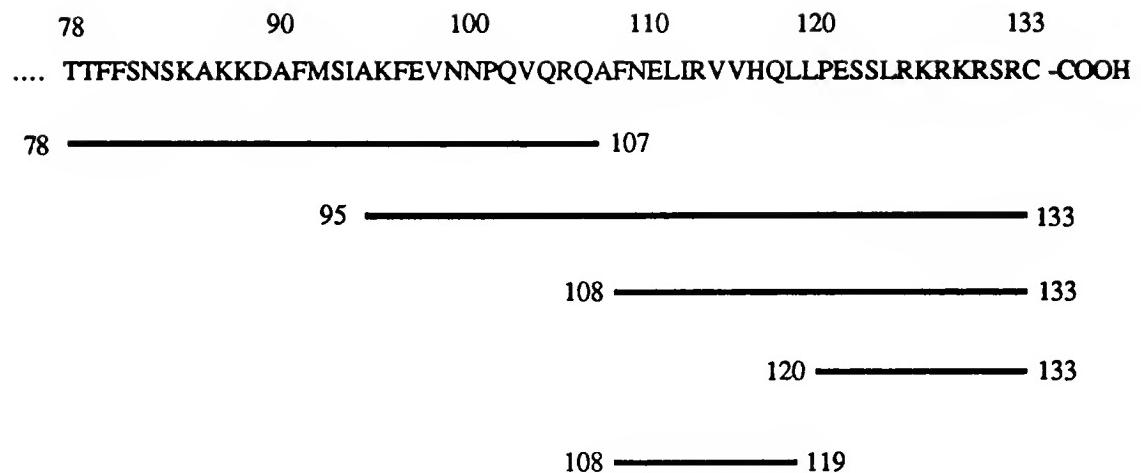


Figure 3-3. C-terminal synthetic peptides of IFN- $\gamma$  used to map the neutralizing epitope specificity of the neutralizing antiserum produced against IFN- $\gamma$ (95-133). The sequence from residues 78-133 is listed on top with the individual peptides represented by lines under the sequence.

Table 3-4. Epitope mapping of the C-terminal specific IFN- $\gamma$  neutralizing antiserum<sup>a</sup> and identification of the neutralizing epitopes.

| Peptide Conc.<br>(mM) | IFN- $\gamma$ titer (units) <sup>b</sup> |                         |                        |                         |                         | 59  |
|-----------------------|--|-------------------------|------------------------|-------------------------|-------------------------|-----|
|                       | IFN- $\gamma$ (95-133)                   | IFN- $\gamma$ (108-133) | IFN- $\gamma$ (78-107) | IFN- $\gamma$ (108-119) | IFN- $\gamma$ (120-133) |     |
| 0.1                   | 300                                      | 100                     | 30                     | 3                       | 30                      | 300 |
| 0.03                  | 300                                      | 100                     | 30                     | 3                       | 30                      | 300 |
| 0.01                  | 300                                      | 100                     | 30                     | 3                       | 30                      | 300 |
| 0.003                 | 300                                      | 100                     | 30                     | 3                       | 30                      | 300 |
| 0.001                 | 300                                      | 100                     | 30                     | 3                       | 30                      | 300 |
| 0.0003                | 300                                      | 100                     | 30                     | 3                       | 10                      | 300 |
| 0.0001                | 100                                      | 60                      | 30                     | 3                       | 6                       | 300 |
| 0.00003               | 10                                       | 6                       | 10                     | 3                       | 3                       | 100 |

<sup>a</sup>Antiserum was used at a dilution of 1/1000.

<sup>b</sup>IFN- $\gamma$  titer in the absence of antiserum was 300 units. In the presence of antiserum the titer was <3 units.

<sup>c</sup>The concentration of the mixture of peptides in the last column reflects the individual peptide concentrations listed in the lefthand column.

and IFN- $\gamma$ (108-119) had a slight inhibitory ability when added separately. However, when the longer sequence was reconstructed with the combination of the shorter peptides, IFN- $\gamma$ (78-107), IFN- $\gamma$ (108-119), and IFN- $\gamma$ (120-133), full inhibitory activity was restored. This suggests that there are multiple neutralizing epitopes involved in this region that can function cooperatively and that the antiserum is probably directed toward several linear sequences.

CD studies of human IFN- $\gamma$  have shown that the molecule is about 66%  $\alpha$ -helix with turn and random regions making up the difference (80). Mouse and human IFN- $\gamma$  share about 40% identical sequence homology (20). About 30% of the amino acid changes are conservative substitutions. Both mouse and human IFN- $\gamma$  possess very similar surface profiles as shown in figure 3-4. The surface profile predicts which regions of a protein may be found on the surface of the molecule. The surface profile takes into account HPLC mobility, segmental mobility and surface accessibility parameters for amino acid residues calculated from model proteins and peptides (121). The overlapping profiles suggest that human and mouse IFN- $\gamma$  are very similar at the structural level. Therefore, it is reasonable that information obtained concerning human IFN- $\gamma$  may be applicable to mouse IFN- $\gamma$ . Due to the overall structural homology between human and mouse IFN- $\gamma$ , mouse IFN- $\gamma$  is likely to contain a similar amount of  $\alpha$ -helix. I was unable to obtain mouse IFN- $\gamma$  in sufficient quantities to measure the CD directly; however, I did measure the CD of the

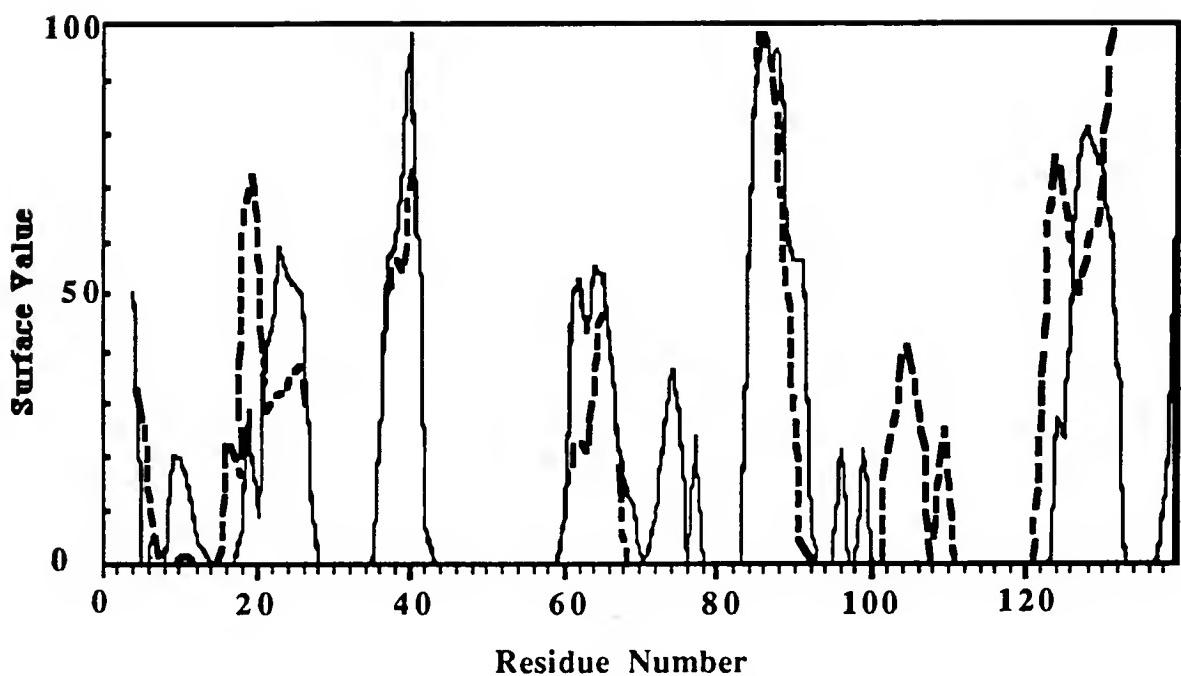


Figure 3-4. Surface profile of mouse IFN- $\gamma$  (dashed lines) and human IFN- $\gamma$  (solid lines).

overlapping synthetic peptides that encompass the entire sequence. CD of the peptides was measured in 25%TFE, a helix stabilizing agent. TFE is commonly used in the CD measurement of synthetic peptides in order to enhance existing structure (11,101-103). Alpha helical structure was observed in all of the peptides characterized by a minimum at 220 nm and a maximum at 190 nm (Figure 3-5). IFN- $\gamma$ (36-60) was not sufficiently soluble in the concentration required for CD measurement due to its hydrophobicity. Although the CD of peptides may differ from that of the native molecule, these results suggest the probability that the peptides form  $\alpha$ -helix and the likelihood that the native molecule is mainly  $\alpha$ -helical.

Based on the above results an overall topology of mouse IFN- $\gamma$  can be pictured. Both the N-terminus and C-terminus participate, in some way, in receptor interaction. The N-terminus, based on direct peptide competition studies can interact with receptor. Additionally, others have shown that when the N-terminal 8-10 residues of human IFN- $\gamma$  are removed by limited proteolysis, functional activity is reduced along with a corresponding loss in  $\alpha$ -helical structure (80). The C-terminus, based on my antibody neutralization data, may also be interacting with receptor or act to stabilize the structure of the N-terminus. Two secondary structure prediction algorithms predict that the molecule contains six  $\alpha$ -helices, no  $\beta$ -sheet, and the remainder turns and random structure (107,108,122). The secondary structure prediction

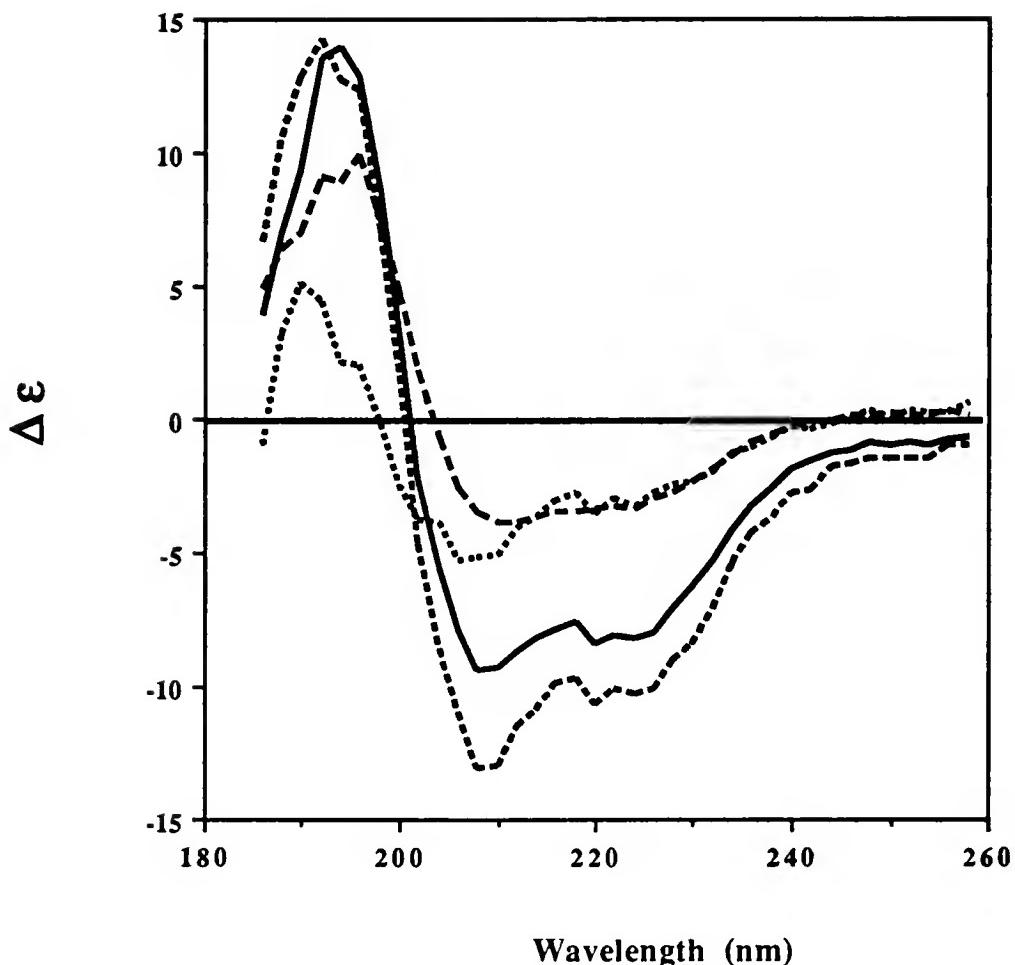


Figure 3-5. CD spectra of overlapping synthetic peptides of IFN- $\gamma$ . Spectra were obtained in 25%TFE as described in Materials and Methods. The spectra for IFN- $\gamma$ (1-39) was provided by H.I. Magazine and was generated under the same conditions as the other peptides. Symbols: IFN- $\gamma$ (1-39) (— — —), IFN- $\gamma$ (54-91) (— — —), IFN- $\gamma$ (78-107) (.....), IFN- $\gamma$ (95-133) (— · — · —).

is shown in figure 3-6. These predictions are in agreement with what is observed with CD studies of human IFN- $\gamma$  (80) and of synthetic peptides of mouse IFN- $\gamma$ . All six of the  $\alpha$ -helices possess some degree of apolar periodicity that is characteristic of a globular protein that can fold into a common tertiary motif called a four- $\alpha$ -helix bundle (123-125). The apolar periodicity forms a hydrophobic ridge on one side of an  $\alpha$ -helix that would allow it to interact with other helices to form a four helix bundle.

Considering these results, a simple three dimensional model of IFN- $\gamma$  was constructed (Figure 3-7). It has become clear that many proteins, although they have different primary sequences, fold into similar tertiary structure motifs (126). Seemingly unrelated proteins have been found to have virtually the same topology (126). A pattern of structural motifs are repeated throughout proteins of known structure. IFN- $\gamma$  and IL-2 share a number of structural components, so the IFN- $\gamma$  tertiary structure was principally patterned after the three dimensional structure of IL-2 which has been derived by X-ray crystallography (127). Therefore, the following similarities form the rationale for basing the structure of IFN- $\gamma$  on the tertiary structure of IL-2. Both proteins are of a similar length. Both proteins are predicted to contain around 60%  $\alpha$ -helix and no  $\beta$ -sheet as determined by predictive algorithms (107,108,122), CD studies of mouse IFN- $\gamma$  synthetic peptides and human IFN- $\gamma$  (80), and by X-ray crystallographic studies for IL-2 (127).

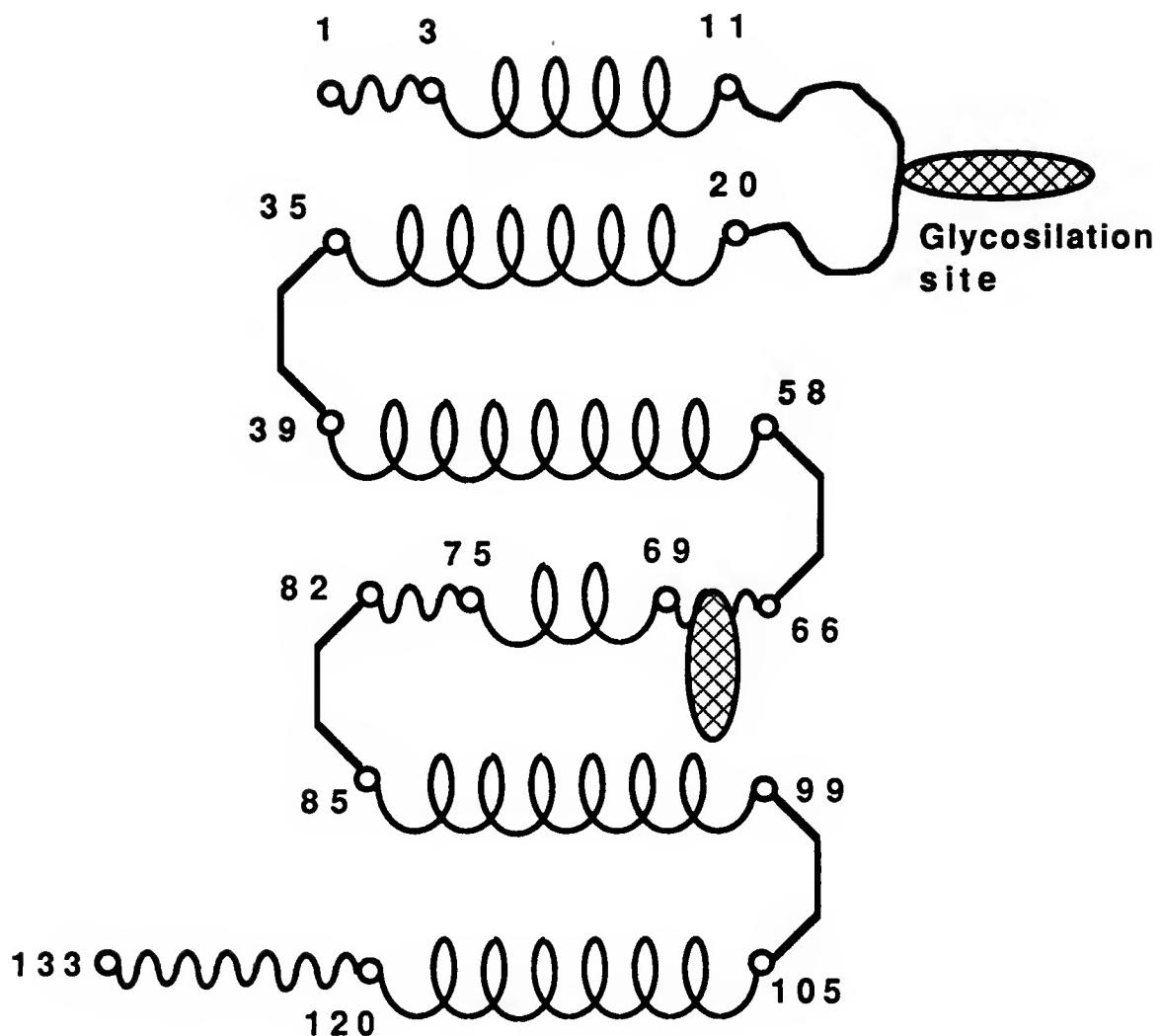


Figure 3-6. Predicted secondary structure of mouse IFN- $\gamma$ . Wavy lines depict random structure, coils represent  $\alpha$ -helix, and lines represent turns and loops. There is a predicted omega loop structure between residues 12-20. Hatched ovals represent glycosylation sites.

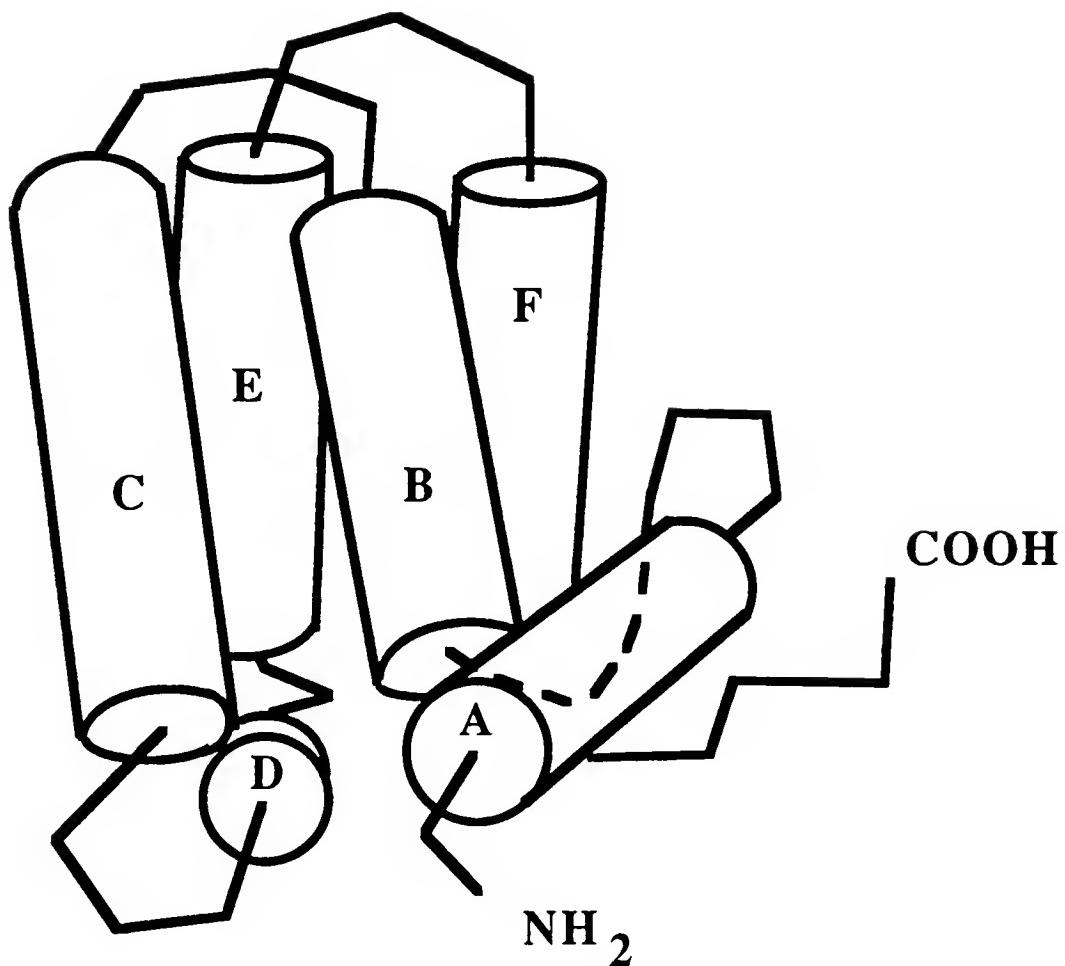


Figure 3-7. Predicted tertiary structure of murine IFN- $\gamma$ . The barrels depict  $\alpha$ -helices and the letters indicate the order of each helix along the sequence from N-terminus to C-terminus.

Interleukin-2 has six helices, one of which contains a proline kink, while IFN- $\gamma$  is predicted to contain six helices. Interleukin-2 possesses an extended loop in the N-terminus between the first and second helix and IFN- $\gamma$  is predicted to have a similar loop (109). There is a predicted amphipathic helix in the C-terminus of IFN- $\gamma$  (Figure 3-8) and a known amphipathic helix in the C-terminus of IL-2 (111). Interleukin-2 folds into a four-helix bundle motif (127). This bundle motif, as well as most of the other structural components of IL-2, were successfully predicted using similar methods as those described here (128).

The predicted tertiary structure of IFN- $\gamma$  follows the backbone of IL-2 closely, with two major exceptions. Interleukin-2 has a disulfide bridge that links the second and fourth turn. Interferon  $\gamma$  has no disulfide bonds. Also, in IL-2, the fifth helix is six residues in length and is shorter than the others. This helix does not participate in the four-helix-bundle and forms a bridge between the fourth and sixth helices. The fourth helix in IFN- $\gamma$  is the shorter, six residue helix. Therefore, the fourth and fifth helices were transposed in the model of IFN- $\gamma$ . The fourth helix forms a bridge between the third and fifth helices. This model fits well with the existing data concerning the structure and function relationship of IFN- $\gamma$  and a list of this information can be found in Table 5. The key feature involves the location of the functional domains. Evidence suggests that the N- and C-terminus are important for

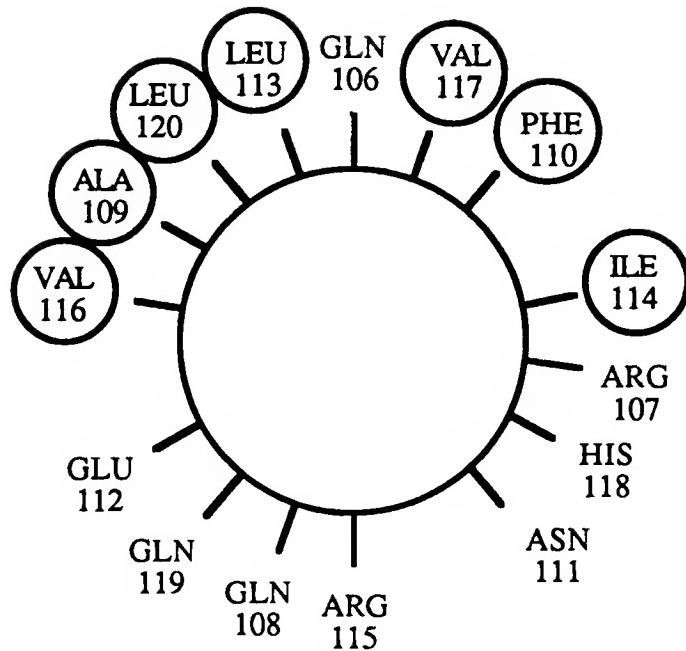


Figure 3-8. Helix wheel depicting amphipathic  $\alpha$ -helix in the C-terminus of mouse IFN- $\gamma$  between residues 106-120. Hydrophobic residues are circled.

Table 3-5. Summary of information used to generate the 3-dimensional model of mouse IFN- $\gamma$ .

|   |  | Reference            |
|---|--|----------------------|
| <u>N-terminal domain</u>  |  |                      |
| N-terminal peptide, IFN- $\gamma$ (1-39), directly blocks IFN- $\gamma$ binding and function  |  | 88                   |
| Polyclonal and monoclonal antibodies directed to the N-terminus neutralize function through the blockage of receptor binding.   |  | 87, 88<br>chapter 2  |
| <u>C-terminal domain</u>  |  |                      |
| Site directed polyclonal antisera to the C-terminus block IFN- $\gamma$ receptor binding and neutralize function.   |  | 4, this work         |
| Truncations of the C-terminus of human IFN- $\gamma$ abrogate function.   |  | 81, 83               |
| <u>Central region</u>   |  |                      |
| Antibodies to the central region of the molecule have no effect on IFN- $\gamma$ activity.  |  | this work            |
| <u>Secondary structure prediction</u>   |  |                      |
| Predicted location of turns: Residues- 16-20, 36-40, 60-64, 81-85, 100-104.   |  | 107, 108,<br>122     |
| The remainder of the the molecule is predicted to form $\alpha$ -helices with some random structure. No $\beta$ -sheet is predicted.                                      |  | 107, 108,<br>122     |
| <u>Circular dichroism</u>   |  |                      |
| Human IFN- $\gamma$ consists of 66% $\alpha$ -helix. No $\beta$ -sheet present.   |  | 80                   |
| Circular dichroism of overlapping synthetic peptides of mouse IFN- $\gamma$ confirm the presence of mainly $\alpha$ -helical structure and the absence of $\beta$ -sheet. |  | this work            |
| <u>Tertiary structure prediction</u>  |  |                      |
| Presence of apolar periodicity noted in all $\alpha$ -helices which suggests the probability of a four- $\alpha$ -helix bundle motif.                                     |  | 123-125              |
| Functional data suggest the N and C-termini are functional domains interacting with the receptor which places them close together.  |  | 87, 88,<br>chapter 2 |
| <u>Structural similarity to IL-2.</u> Reasonable to base model on backbone of IL-2 three dimensional structure.   |  |                      |

receptor binding and are in close proximity. In this model the N- and C-terminal helices are close together and both could presumably interact with receptor. It has been postulated that the functional unit of IFN- $\gamma$  exists as a dimer (75-77) or tetramer (78) in solution. However, IFN- $\gamma$  has been shown to have activity when isolated in its monomer form (75). Thus, the functional form of IFN- $\gamma$  has yet to be resolved. For simplicity, the model has been built based on a single polypeptide chain. It is difficult to speculate, based on existing data, which structural features might be involved in the formation and maintenance of multimer IFN- $\gamma$ . One additional observation involves the glycosylation sites on the molecule. The glycosylation sites are included in secondary structure prediction in figure 3-6. The sites would fall in the N-terminal loop region and in the turn between helix C and helix D on the tertiary structure prediction (figure 3-7). These sites are exposed and accessible to sugar residues in the model.

Although other orientations are possible, this model was chosen as the most probable based on existing data. Additionally, the overall structural features, the four- $\alpha$ -helix bundle and the N- and C-termini exposed and together, are the most important characteristics. This model provides a working base that will be useful in the design of future studies involving this important lymphokine.

CHAPTER 4  
SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

IFN- $\gamma$  is an important immunoregulatory lymphokine that is involved in a diverse number of activities related to the immune response. By understanding its mechanism of action one may be able to modulate its activities in some way that may be beneficial. Possibly, its many activities may be dissected and the generation of agonists or antagonists that differentially modulate IFN- $\gamma$  activity may be able to be designed. To achieve these ambitious goals one must first understand, at the molecular level, what structures are involved in IFN- $\gamma$  function. There has been a great deal of work carried out in this area but much information is lacking. The work that has been described within this dissertation focuses on the identification and characterization of functional domains of mouse IFN- $\gamma$  through the use of monoclonal and polyclonal antibodies and the synthetic peptide approach.

A mAb neutralizes IFN- $\gamma$  by blocking IFN- $\gamma$  binding to receptor, so a description of the epitope to which it binds may also give insight to this IFN- $\gamma$  binding domain. The epitope specificity of this mAb was mapped with synthetic peptides to the N-terminus of the molecule and found to be contained between residues 3-14 of IFN- $\gamma$ . This region is predicted to contain an amphipathic  $\alpha$ -helix between residues

3-11. The residues at positions three, four and five were shown to be important when analogs with nonconservative substitutions that abrogated the amphipathicity of the  $\alpha$ -helix were found not to bind the mAb. A truncated peptide that lacked tyrosine at position 14 also lacked binding ability. Circular dichroism analysis confirmed the presence of  $\alpha$ -helix in the peptides in the presence of TFE but not in water alone, suggesting that secondary structure is not important for epitope structure, however secondary structure is certainly present in the intact molecule. The epitope can be described as a linear discontinuous epitope because of the lack of secondary structure requirement and the fact that it is longer than most continuous epitopes (8-10 residues). The charge and hydropathy of the residues contained in the N-terminal part of the amphipathic helix as well as the tyrosine at position 14 are critical to the epitope. These studies have characterized the site of interaction of a mAb that binds to an important region on IFN- $\gamma$ .

It has been previously demonstrated that a peptide of IFN- $\gamma$  that corresponds to the first 39 residues of the molecule has the ability to block IFN- $\gamma$  function and receptor binding (88). This peptide does not have agonist properties and interacts with the receptor with 1/1000th affinity as IFN- $\gamma$ . Therefore, other regions of IFN- $\gamma$  must be important in receptor binding. Studies have suggested that the C-terminus may also be involved but have not been conclusive (81-84,87). Additionally, the remainder of the molecule has

not been examined. Long overlapping peptides that correspond to the entire sequence of IFN- $\gamma$  were synthesized. Only IFN- $\gamma$ (1-39), as shown previously (88), had the ability to compete for receptor. Antibodies raised to the peptides were able to bind to IFN- $\gamma$  but only antibodies to the N-terminus and to the C-terminus could neutralize IFN- $\gamma$  activity. These neutralizing antibodies blocked IFN- $\gamma$  receptor binding. The neutralizing epitopes of the C-terminal specific antiserum are found along the entire region as evidenced by peptide/antibody competition studies. Only peptides corresponding to the entire C-terminus or combinations of peptides that reconstruct the C-terminus with shorter sequences could effectively compete with IFN- $\gamma$  for antiserum binding to block IFN- $\gamma$  neutralization. Thus, both the N-terminus and the C-terminus are involved in binding.

Data presented here support a two domain model for IFN- $\gamma$  binding to its receptor. The N-terminus and C-terminus are the only regions shown to be directly involved in IFN- $\gamma$  receptor binding and since only one receptor protein has been identified (17-21), the N-terminus and C-terminus are probably relatively close together in the active IFN- $\gamma$  molecule. Circular dichroism studies of human IFN- $\gamma$  show that the molecule is 66%  $\alpha$ -helix and no  $\beta$ -sheet. Circular dichroism spectra of overlapping synthetic peptides of mouse IFN- $\gamma$  confirm the ability of these peptides to form  $\alpha$ -helical conformation with the absence of  $\beta$ -sheet. Predictive algorithms divide the molecule into six  $\alpha$ -helices and five

turns and each of the helicies has a degree of apolar periodicity. These characteristics allow for the potential that the IFN- $\gamma$  forms a four-helix-bundle motif with the N-terminus and C-terminus close together forming receptor binding domains. The internal  $\alpha$ -helices are hydrophobic and buried within the structure. Ideally, x-ray crystallographic data would be most helpful in understanding IFN- $\gamma$ . This model is based on the similarities that exists between IFN- $\gamma$  and IL-2. IL-2 is comprised of seven  $\alpha$ -helices with a four helix bundle motif. The N-terminus and C-terminus are also in close proximity.

These studies have described in further detail and have advanced the understanding of the structure/function relationship of IFN- $\gamma$ . They describe a molecule that interacts with its receptor with two domains, one in the N-terminus and one in the C-terminus. The role of the N-terminus is clear. Based on previous studies the N-terminus participates directly in receptor binding (88). The role of the C-terminus is, as yet, unclear. It may also participate directly in binding or may act to stabilize another part of the molecule. Further study is required to determine its role.

Future studies should concentrate on the study of IFN- $\gamma$  using molecular biology techniques. In the past, the usefulness of this approach has been minimal due to the lack of a rational basis for site-directed mutations. Random mutations have yielded molecules with reduced activity but

have resulted in little understanding as to the reason for the change in function. A more directed approach is required where deletions and substitutions are targeted toward areas shown to be involved in binding. The synthetic peptide and antibody approach used in this and other studies have identified regions of the IFN- $\gamma$  molecule for site-directed mutations. With the information at hand we are in a position to begin engineering the IFN- $\gamma$  molecule, attempting to design agonists and antagonists that can modulate its function. Another area of potential interest concerns the molecular basis for species specificity of the IFN- $\gamma$  molecule. The synthetic peptide approach would also be useful in determining critical residues of IFN- $\gamma$  that are responsible for species specificity. Chimeric peptides of human and mouse IFN- $\gamma$  could be designed that contain sequences in common to the two species as well as residues unique to each. These peptides could then be assessed for their relative abilities to block IFN- $\gamma$  binding to receptor. With this information, recombinant molecules could then be constructed to further study the basis for species specificity of IFN- $\gamma$ .

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## BIOGRAPHICAL SKETCH

Michael Andrew Jarpe was born on July 19, 1962, to Jay Stephen and Marion Blair Jarpe in St. Joseph, Michigan. He has one sister, Jennifer, and four brothers, Stephen, Geoff, Matthew, and Andrew. After moving to New Mexico with his family in 1975, Michael grew up in a rural setting which had a strong influence on his development. He graduated from Los Lunas High School in 1980 and enrolled in New Mexico Institute of Mining And Technology, a small science oriented college, to obtain a degree in biology. There he met and married his wife, Alyssa Joy Casper, on June 17, 1984. He earned his B.S. in biology in December, 1984.

In August, 1985, Michael entered the Ph.D program in immunology in the department of Pathology and Laboratory Medicine at the University of Florida. He began his study under Dr. Stephen W. Russell and subsequently moved to the laboratory of Dr. Howard M. Johnson in August, 1987. After receiving his degree in August, 1990, Michael will be pursuing the study of IFN- $\gamma$  at the molecular biology level with Dr. Johnson in a postdoctoral position.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Howard M. Johnson

Howard M. Johnson, Chair  
Graduate Research Professor  
of Pathology and  
Laboratory Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

J. R. Dankert

John R. Dankert  
Assistant Professor of  
Veterinary Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Paul A. Hargrave

Paul A. Hargrave  
Professor of Biochemistry  
and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Lindsey M. Hutt-Fletcher

Lindsey M. Hutt-Fletcher  
Associate Professor of  
Pathology and Laboratory  
Medicine

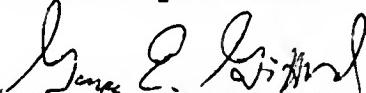
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Ammon B. Peck

Associate Professor of  
Pathology and Laboratory  
Medicine

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy

August, 1990

  
Joyce E. Griffith

Dean, College of Medicine

  
Madelyn Hackhart

Dean, Graduate School

UNIVERSITY OF FLORIDA



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